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(54) Title: **ANTI-CCR5 ANTIBODY**

(57) **Abstract:** The invention is directed an anti-CCR5 antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising an expression product of either a plasmid designated pVgl:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVgl:HuPRO140 (mutB+D+I)-VH (ATCC Deposit Designation PTA-4099) or a fragment thereof which binds to CCR5 on the surface of a human cell.

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ANTI-CCR5 ANTIBODY

This application is a continuation-in-part of and claims the priority of U.S. Application Serial No. 10/081,128, filed February 22, 2002, the contents of which are hereby incorporated by reference into this application.

Throughout this application, various publications are referenced by Arabic numerals. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Background of the Invention

Human immunodeficiency virus type 1 (HIV-1) induces viral-to-cell membrane fusion to gain entry into target cells (8, 15, 66). The first high-affinity interaction between the virion and the cell surface is the binding of the viral surface glycoprotein gp120 to the CD4 antigen (13, 30, 41, 42). This in turn induces conformational changes in gp120, which enable it to interact with one of several chemokine receptors (4, 5, 21, 36). The CC-chemokine receptor CCR5 is the major co-receptor for macrophage-tropic (R5) strains, and plays a crucial role in the sexual transmission of HIV-1 (4, 5, 21, 36). T cell line-tropic (X4) viruses use CXCR4 to enter target cells, and usually, but not always, emerge late in disease progression or as a consequence of virus propagation in tissue culture (4, 5, 21, 36). Some primary HIV-1 isolates are dual-tropic (R5X4) since they can use both co-receptors, though not always with the same efficiency (11, 57). Mutagenesis studies coupled with the resolution of the gp120 core crystal structure demonstrated that the co-receptor-

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binding site on gp120 comprises several conserved residues (32, 53, 65).

It has been demonstrated that tyrosines and negatively charged residues in the amino-terminal domain (Nt) of CCR5 are essential for gp120 binding to the co-receptor, and for HIV-1 fusion and entry (6, 18, 20, 22, 28, 31, 52, 54). Residues in the extracellular loops (ECL) 1-3 of CCR5 were dispensable for co-receptor function, yet the CCR5 inter-domain configuration had to be maintained for optimal viral fusion and entry (24). This led to the conclusion either that gp120 forms interactions with a diffuse surface on the ECLs, or that the Nt is maintained in a functional conformation by bonds with residues in the ECLs. Studies with chimeric co-receptors and anti-CCR5 monoclonal antibodies have also shown the importance of the extracellular loops for viral entry (5, 54, 64).

Molecules that specifically bind to CCR5 and CXCR4 and block interactions with their ligands are a powerful tool to further probe the structure/function relationships of the co-receptors. Characterizing such compounds could also assist in designing effective therapeutic agents that target co-receptor-mediated steps of viral entry. Inhibitors of CCR5 or CXCR4 co-receptor function identified to date are diverse in nature and include small molecules, peptides, chemokines and their derivatives, and monoclonal antibodies (mAbs). The mechanisms of action of the small molecules that block entry by interfering with CXCR4 co-receptor function are not well understood (17, 49, 55, 68). One such inhibitor, the anionic small molecule AMD3100, depends on residues in ECL2 and the fourth trans-membrane (TM) domain of CXCR4 to inhibit viral entry, but it is not clear whether it does so by disrupting gp120 binding to CXCR4 or post-binding steps

Summary of the Invention:

This invention provides an anti-CCR5 antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO 140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell.

This invention also provides an anti-CCR5 antibody comprising two light chains, each chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6, and two heavy chains, each heavy chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:9.

This invention also provides an anti-CCR5 antibody comprising two light chains, each chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6, and two heavy chains, each heavy chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:12.

This invention also provides an isolated nucleic acid encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6. In the subject embodiment, the nucleic acid comprises the sequence set forth in SEQ ID NO:5.

This invention also provides an isolated nucleic acid

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encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:9. In the subject embodiment, the nucleic acid comprises the sequence set forth in SEQ ID NO:8.

This invention also provides an isolated nucleic acid encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:12. In the subject embodiment, the nucleic acid comprises the sequence set forth in SEQ ID NO:11.

This invention also provides a composition comprising at least one anti-CCR5 antibody, or a fragment thereof, as described above, together with a carrier.

This invention also provides a composition comprising the anti-CCR5 antibody, or a fragment thereof, having attached thereto a material such as a radioisotope, a toxin, polyethylene glycol, a cytotoxic agent and/or a detectable label.

This invention also provides a method of inhibiting infection of a CD4+ cell which comprises contacting the CD4+ cell with an antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody which binds to CCR5 on the surface of a CD4+ cell, in an amount and under conditions such that fusion of HIV-1 or an HIV-1-infected cell to the CD4+ cell is inhibited, thereby inhibiting HIV-1 infection of the CD4+ cell.

This invention also provides a method of treating a subject afflicted with HIV-1 which comprises administering to the subject an effective HIV-1 treating dosage of an anti-CCR5 antibody comprising (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell, under conditions effective to treat the HIV-1-infected subject.

This invention also provides a method of preventing a subject from contracting an HIV-1 infection which comprises administering to the subject an effective HIV-1 infection-preventing dosage amount of an anti-CCR5 antibody comprising (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell, under conditions effective to prevent the HIV-1, infection in the subject.

This invention also provides an anti-CCR5 antibody conjugate comprising an anti-CCR5 antibody which comprises (i) two light chains, each light chain

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comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody which binds to CCR5 on the surface of a human cell, conjugated to at least one polymer.

This invention also provides a method of inhibiting infection of a CCR5+ cell by HIV-1 comprising administering to a subject at risk of HIV-1 infection the above-described conjugate in an amount and under conditions effective to inhibit infection of CCR5+ cells of the subject by HIV-1.

This invention also provides a method of treating an HIV-1 infection in a subject comprising administering the above-described conjugate to an HIV-1-infected subject in an amount and under conditions effective to treat the subject's HIV-1 infection.

This invention also provides a transformed host cell comprising at least two vectors, at least one vector comprising a nucleic acid sequence encoding heavy chains of an anti-CCR5 antibody, and at least one vector comprising a nucleic acid sequence encoding light chains of the anti-CCR5 antibody, wherein the anti-CCR5 antibody comprises two heavy chains having the amino acid sequence set forth in SEQ ID NO:9, and two light chains having the amino acid sequence set forth in SEQ ID NO:6.

This invention also provides a transformed host cell comprising at least two vectors, at least one vector

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comprising a nucleic acid sequence encoding heavy chains of an anti-CCR5 antibody, and at least one vector comprising a nucleic acid sequence encoding light chains of the anti-CCR5 antibody, wherein the anti-CCR5 antibody comprises two heavy chains having the amino acid sequence set forth in SEQ ID NO:12 and two light chains having the amino acid sequence set forth in SEQ ID NO:6.

This invention also provides a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:9.

This invention also provides a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:12.

This invention also provides a process for producing an anti-CCR5 antibody which comprises culturing a host cell containing therein (i) a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099) under conditions permitting the production of an antibody comprising two light chains encoded by the plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation

PTA-4097) and two heavy chains encoded either by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or by the plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), so as to thereby produce an anti-CCR5 antibody.



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This invention also provides a process for producing an anti-CCR5 antibody which comprises a) transforming a host cell with (i) a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097) and (ii) either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), and b) culturing the transformed host cell under conditions permitting production of an antibody comprising two light chains encoded by the plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097) and two heavy chains encoded either by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or by the plasmid designated pVg1HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), so as to thereby produce an anti-CCR5 antibody.

This invention also provides a kit for use in a process of producing an anti-CCR5 antibody. The kit comprises a) a vector comprising a nucleic acid sequence encoding a light chain of an anti-CCR5 antibody, wherein the light chain comprises the amino acid sequence set forth in SEQ ID NO:6, and b) a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:9, or a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:12.

Brief Description of the Figures:Figure 1:Binding of anti-CCR5 monoclonal antibodies to CCR5<sup>+</sup> cells:

Flow cytometry was used to detect CCR5 protein expression on the surface of L1.2-CCR5<sup>+</sup> cells and freshly isolated, PHA/IL-2-stimulated PBMC. Cells were incubated with saturating concentrations of each mAb, which were detected with a PE-labeled anti-mouse IgG reporter antibody. Results from a representative experiment are shown. Results for each mAb are expressed both in mean fluorescence intensities (m.f.i.) and in % gated cells. Since PA8-PA12 and PA14 are all of the IgG1 subclass, their m.f.i. are directly comparable. 2D7 is an IgG2a.

Figure 2:CI values for different combinations of mAbs and viral inhibitors:

Experiments like those described in the legend of Fig. 7 were performed for different combinations of viral entry inhibitors. Anti-CCR5 mAbs were tested in combination with each other, CC-chemokines, and CD4-IgG2, which inhibits HIV-1 attachment to target cells. The PA11 and PA12 concentration range was 0-250 µg/ml; the 2D7 and PA14 concentration range was 0-25 µg/ml; the RANTES concentration range was 0-250 ng/ml; the CD4-IgG2 concentration range was 0-25 µg/ml. The concentrations of single-agents or their mixtures required to produce 50% and 90% inhibition of fusion or entry were quantitatively compared in a term known as the Combination Index (CI).

Figure 3:

IC<sub>50</sub> values for inhibition of cell-cell fusion, viral entry and gp120/sCD4 binding by anti-CCR5 mAbs:

For comparative purposes we have summarized the IC<sub>50</sub> values obtained in the different assays that the anti-CCR5 mAbs were tested in. IC<sub>50</sub> values were only calculated for mAbs that could inhibit >90% of fusion, entry or binding.

Figure 4:

Epitope mapping of anti-CCR5 mAbs:

A two color staining protocol was used to assess binding of mAbs to mutant CCR5 proteins, tagged at the C-terminus with the HA peptide. HeLa cells expressing CCR5 point mutants were incubated with saturating concentrations of each mAb followed by detection with a PE-labeled anti-mouse IgG. Cell surface co-receptor expression was measured by double-staining of the cells with a FITC labeled anti-HA mAb. The four grids correspond to the four extracellular domains of CCR5. The first row of every grid indicates the amino acid sequence of the corresponding CCR5 extracellular domain (SEQ ID NOS: 1-4). Binding of anti-CCR5 mAbs to the alanine mutant of each residue is expressed as a percentage of binding to wild-type CCR5, as described in Materials and Methods.

Figure 5:

Inhibition of calcium mobilization into CCR5<sup>+</sup> cells by anti-CCR5 mAbs:

L1.2-CCR5<sup>+</sup> cells were loaded with Indo-1AM and stimulated sequentially with an anti-CCR5 mAb or PBS, followed with RANTES (a). Fluorescence changes were measured with a spectrofluorometer and the tracings are from a representative

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experiment. Calcium flux inhibition by PA14 and 2D7 was tested for a wide range of mAb concentrations (b). Results are plotted as % inhibition of calcium influx =  $[1 - (\text{relative fluorescence in the presence of mAb} \div \text{relative fluorescence in the absence of mAb})] \times 100\%$ , and are means of values from three independent experiments.

Figure 6:

Inhibition of CCR5 co-receptor function by anti-CCR5 mAbs:

Inhibition of cell-cell fusion by anti-CCR5 mAbs was tested in the RET assay (a). 0-250µg/ml of PA8-PA12, or 0-25µg/ml of PA14 or 2D7, were added to a mix of HeLa-Env<sub>JR-FL</sub> and PM1 cells, labeled with F18 and R18 respectively. Fluorescence RET was measured after 4h of incubation. Results are mean values from three independent experiments and are expressed as % inhibition of fusion =  $[1 - (\% \text{ RET in the presence of mAb} \div \% \text{ RET in the absence of mAb})] \times 100\%$ . Inhibition of HIV-1 entry by anti-CCR5 mAbs was tested in a single round of replication luciferase based entry assay (b). U87-CD4<sup>+</sup>CCR5<sup>+</sup> cells were infected with NLluc<sup>+</sup>env<sup>+</sup> reporter virus carrying the JR-FL envelope in the presence of 0-250µg/ml of PA8-PA12, or 0-25µg/ml PA14 or 2D7. Luciferase activity (relative light units, r.l.u.) was measured in cell lysates 72h post-infection. Results are from a representative experiment and are expressed as % inhibition of entry =  $[1 - (\text{r.l.u. in the presence of mAb} \div \text{r.l.u. in the absence of mAb})] \times 100\%$ . Binding of biotinylated [b] gp120, sCD4 and b-gp120-CD4 complexes to L1.2-CCR5<sup>+</sup> cells (c). Strong binding is observed when gp120 derived from the R5 virus

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HIV-1<sub>JR-FL</sub> is complexed with an equimolar amount of sCD4. No binding is observed in the absence of sCD4 or for gp120 derived from the X4 virus HIV-1<sub>LA1</sub>. Background binding to CCR5- L1.2 cells has been subtracted from all curves. Inhibition of gp120/sCD4 binding to L1.2-CCR5<sup>+</sup> cells was tested in the presence of varying concentrations of each antibody (d). Cells were pre-incubated in 96-well plates with an anti-CCR5 mAb followed by an incubation with a saturating concentration of biotinylated gp120/sCD4. Finally, binding of PE-labeled streptavidin to cells was measured using a fluorescence plate reader. Results are from a representative experiment and are expressed as % inhibition of gp120/sCD4 binding =  $[1 - (\text{m.f.i. in the presence of mAb} / \text{m.f.i. in the absence of mAb})] \times 100\%$ .

Figure 7:

Synergistic inhibition of cell-cell fusion by PA12 and 2D7:

Dose-response curves were obtained for the mAbs used individually and in combination. 0-50µg/ml of PA12, 0-25µg/ml 2D7, or a combination of the two in a 2:1 ratio, were added to a mix of HeLa-Env<sub>JR-FL</sub><sup>+</sup> and PM1 cells, labeled with R18 and F18 respectively. Fluorescence RET was measured after 4 hours of incubation. Results are expressed as % inhibition of fusion and are the means of values from three independent experiments. Data were analyzed using the median effect principle, which can be written

$$f = 1 / [1 + (K/c)^m] \quad (1)$$

where f is the fraction affected/inhibited, c is concentration, K is the concentration of agent required to produce the median effect, and m is an

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empirical coefficient describing the shape of the dose-response curve. Equation (1) is a generalized form of the equations describing Michaelis-Menton enzyme kinetics, Langmuir adsorption isotherms, and Henderson-Hasselbalch ionization equilibria, for which  $m = 1$ . In the present case,  $K$  is equal to the  $IC_{50}$  value.  $K$  and  $m$  were determined by curve-fitting the dose-response curves and Equation (1) was rearranged to allow calculation of  $c$  for a given  $f$ . The best-fit parameters for  $K$  and  $c$  are  $8.8\mu\text{g/ml}$  and  $0.54$  for PA12,  $0.36\mu\text{g/ml}$  and  $0.68$  for 2D7, and  $0.11\mu\text{g/ml}$  and  $1.1$  for their combination. These curves are plotted and indicate a reasonable goodness-of-fit between experiment and theory.

Figure 8:

This figure shows the amino acid sequence of the light chain variable region of a humanized version of mouse anti-CCR5 antibody PA14 (SEQ ID NO: 6) and the nucleic acid sequence encoding the same (SEQ ID NO: 5), in accordance with the invention. SEQ ID NO: 7 identifies the region of SEQ ID NO: 5 which codes for the amino acid sequence set forth in SEQ ID NO:6. This light chain variable region is present in the antibodies designated herein as PRO 140 #1 and #2. The complementarity-determining regions ("CDRs") are underlined.

Figure 9:

This figure shows the amino acid sequence of a first heavy chain variable region of a humanized version of mouse anti-CCR5 antibody PA14 (SEQ ID NO:9), and the nucleic acid sequence encoding the same (SEQ ID NO:8), in accordance with the invention. SEQ ID NO:10 identifies the region of SEQ ID NO:8 that codes for the amino acid sequence

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set forth in SEQ ID NO:9. This heavy chain variable region is present in the antibody designated herein as PRO 140 #2. The CDRs are underlined.

Figure 10:

This figure shows the amino acid sequence of a second heavy chain variable region of a humanized version of mouse humanized anti-CCR5 antibody PA14 (SEQ ID NO:12) and the nucleic acid sequence encoding the same (SEQ ID NO:11) in accordance with the invention. SEQ ID NO:13 identifies the region of SEQ ID NO:11 that codes for the amino acid sequence set forth in SEQ ID NO:12. This heavy chain variable region is present in the antibody designated herein as PRO 140 #1. The CDRs are underlined.

Figure 11:

Single-Dose of humanized CCR5 antibody potentially reduces viral loads in vivo:

SCID mice were reconstituted with normal human PBMC and infected with HIV-1<sub>JR-CSF</sub>. When a viral steady state was reached, the animals were treated with a single 1 milligram i.p. dose of humanized CCR5 antibody (PRO 140) or isotype control antibody and monitored for plasma HIV RNA (Roche Amplicor Assay).

Figure 12:

Sustained Reduction in Viral Load:

SCID mice were reconstituted with normal human PBMC and infected with HIV-1<sub>JR-CSF</sub>. When a viral steady state was reached, the animals were treated i.p. with 0.1 mg doses of humanized CCR5 antibody (PRO140) every three days and monitored for plasma HIV RNA (Roche Amplicor Assay).

Figure 13:

Demonstrates that there was no depletion of lymphocytes with the use of the CCR5 antibody (PRO 140) prepared in accordance with the invention.

Figure 14:

Humanized CCR5 Antibody (PRO140) Potently Blocks CCR5-mediated HIV-1 Cell-Cell Fusion.

Murine CCR5 antibody was humanized using the method of complementarity-determining region (CDR) grafting and framework substitutions. Humanized CCR5 antibodies (PRO 140 #1 and PRO 140 #2) were expressed in Sp2/0 cells, purified by protein A chromatography and tested for the ability to block replication of HIV-1<sub>JR-FL</sub> env-mediated membrane fusion as described (Litwin, et al., J. Virol., 70:6437, 1996).

Figure 15:

Humanized CCR5 Antibody (PRO 140) Mediates Potent, Subtype-Independent Inhibition of HIV-1.

CCR5 Antibodies (Pro 140 #1 and #2) according to the invention were tested for the ability to block replication of wild-type HIV-1 in peripheral blood mononuclear cells (PBMCs) as described (Trkola et al., J. Virol., 72:396, 1998). The extent of viral replication was measured by assaying the p24 antigen content of 7-day PBMC culture supernatants.

Figure 16:

This figure provides a map of plasmid pVK-HuPRO140 encoding the light plasmid chain variable region shown in Figure 8 as well as the human Kappa constant regions as described in Co et al., J. Immunol., 148:1149, 1992.



Figure 17:

This figure provides a map of plasmid pVg4-HuPRO140 HG2 encoding the heavy chain variable region shown in Figure 9 as well as the human heavy chain constant regions, CH1, hinge, CH2, and CH3, of human IgG4 as described in Co et al, Supra.

Figure 18:

This figure provides a map of plasmid pVg4-HuPRO140 (mut B+D+I) encoding the heavy chain variable region shown in Figure 10 as well as the human heavy chain constant regions, CH1, hinge, CH2, and CH3, of human IgG4 as described in Co et al, Supra.

Figure 19Hu PRO140 Blocks HIV-1 But Not RANTES Signaling

PRO140 antibodies according to the invention were tested for the ability to block RANTES-induced calcium mobilization in L1.2-CCR5 cells (Olson, et al., J.Virol., 72:396, 1998). This figure shows that a humanized CCR5 antibody (huPRO140) blocks HIV-1 but not RANTES signaling.

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Detailed Description of the invention:

The plasmids designated as HuPRO140-VK, HuPRO140 (mut+B+D+I)-VH, and HuPRO140 HG2-VH, which are referred to in figures 16, 18, and 17 as pVK-HuPRO140, pVg4-HuPRO140 (mut B+D+I) and pVg4-HuPRO140 HG2, respectively, were deposited with the American Type Culture Collection, Manassas, Va., U.S.A. 20108 on February 22, 2002, under ATCC Accession Nos. PTA 4097, PTA 4099 and PTA 4098 respectively. These deposits were made pursuant to the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure (Budapest Treaty).

This invention provides a composition for inhibiting HIV-1 infection comprising at least two compounds in synergistically effective amounts for inhibiting HIV-1 infection, wherein at least one of the compounds prevents with the productive interaction between HIV-1 and an HIV-1 fusion co-receptor.

As used herein, "composition" means a mixture. The compositions include but are not limited to those suitable for oral, rectal, intravaginal, topical, nasal, ophthalmic, or parenteral administration to a subject. As used herein, "parenteral" includes but is not limited to subcutaneous, intravenous, intramuscular, or intrasternal injections or infusion techniques.

As used herein, "HIV-1" means the human immunodeficiency virus type-1. HIV-1 includes but is not limited to extracellular virus particles and the forms of HIV-1 found in HIV-1 infected cells.

As used herein, "HIV-1 infection" means the introduction of HIV-1 genetic information into a target

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cell, such as by fusion of the target cell membrane with HIV-1 or an HIV-1 envelope glycoprotein' cell. The target cell may be a bodily cell of a subject. In the preferred embodiment, the target cell is a bodily cell from a human subject.

As used herein, "inhibiting HIV-1 infection" means the reduction of the amount of HIV-1 genetic information introduced into a target cell population as compared to the amount that would be introduced without said composition.

As used herein, "compound" means a molecular entity, including but not limited to peptides, polypeptides, and other organic or inorganic molecules and combinations thereof.

As used herein, "synergistically effective" means that the combined effect of the compounds when used in combination is greater than their additive effects when used individually.

As used herein, "productive interaction" means that the interaction of HIV-1 and the HIV-1 co-receptor would lead to the fusion of said HIV-1 or HIV-1 envelope glycoprotein' cell and the membrane bearing the co-receptor.

As used herein, "prevents the productive interaction" means that the amount of interaction is reduced as compared to the amount that would occur without the compound. The interactions may be prevented by masking or altering interactive regions on the co-receptor or HIV-1 or by altering the expression, aggregation, conformation, or association state of the co-receptor.

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As used herein, "HIV-1 fusion co-receptor" means a cellular receptor that mediates fusion between the target cell expressing the receptor and HIV-1 or an HIV-1 envelope glycoprotein' cell. HIV-1 fusion co-receptors include but are not limited to CCR5, CXCR4 and other chemokine receptors.

This invention also provides a composition which inhibits fusion of HIV-1 or an HIV-1 envelope glycoprotein' cell to a target cell, comprising at least two compounds in synergistically effective amounts for inhibiting fusion of HIV-1 or an HIV-1 envelope glycoprotein' cell to a target cell, wherein at least one of the compounds prevents the productive interaction between HIV-1 and an HIV-1 fusion co-receptor.

As used herein, "fusion" means the joining or union of the lipid bilayer membranes found on mammalian cells or viruses such as HIV-1. This process is distinguished from the attachment of HIV-1 to a target cell. Attachment is mediated by the binding of the HIV-1 exterior glycoprotein to the human CD4 receptor, which is not a fusion co-receptor.

As used herein, "inhibits" means that the amount is reduced as compared with the amount that would occur without the composition.

As used herein, "target cell" means a cell capable of being infected by or fusing with HIV-1 or HIV-1 infected cells.

As used herein, "chemokine" means a cytokine that can stimulate leukocyte movement. They may be characterized as either cys-cys or cys-X-cys depending on whether the two amino terminal cysteine residues are immediately

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adjacent or separated by one amino acid. It includes but is not limited to RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , SDF-1 or another chemokine which blocks HIV-1 infection.

In one embodiment of the above compositions, the co-receptor is a chemokine receptor. In the preferred embodiment of the above compositions, the chemokine receptor is CCR5 or CXCR4. Several other chemokine and related receptors are known to function as HIV co-receptors including but not limited to CCR2, CCR3, CCR8, STRL33, GPR-15, CX3CR1 and APJ (69).

As used herein, "chemokine receptor" means a member of a homologous family of seven-transmembrane spanning cell surface proteins that bind chemokines.

As used herein, "CCR5" is a chemokine receptor which binds members of the C-C group of chemokines and whose amino acid sequence comprises that provided in Genbank Accession Number 1705896 and related polymorphic variants.

As used herein, "CXCR4" is a chemokine receptor which binds members of the C-X-C group of chemokines and whose amino acid sequence comprises that provided in Genbank Accession Number 400654 and related polymorphic variants.

In one embodiment of the above compositions, at least one of the compounds is a nonpeptidyl molecule. In one embodiment, the nonpeptidyl molecule is the bicyclam compound AMD3100. (16).

As used herein, "nonpeptidyl molecule" means a molecule that does not consist in its entirety of a linear sequence of amino acids linked by peptide bonds. A nonpeptidyl molecule may, however, contain one or

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more peptide bonds.

In one embodiment of the above compositions, at least one of the compounds is an antibody. In one embodiment, the antibody is a monoclonal antibody. In another embodiment, the antibody is a anti-chemokine receptor antibody. In one embodiment, the antibody is an anti-CXCR4 antibody. In a further embodiment, the anti CXCR4 antibody is 12G5. (43). In a preferred embodiment, the antibody is an anti-CCR5 antibody. The anti-CCR5 antibody includes but is not limited to PA8, PA9, PA10, PA11, PA12, PA14 and 2D7. In this composition the compounds are in an appropriate ratio. The ratio ranges from 1:1 to 1000:1.

The monoclonal antibodies PA8, PA9, PA10, PA11, PA12 and PA14 were deposited pursuant to and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 on December 2, 1998 under the following Accession Nos.: ATCC Accession No. HB-12605 (PA8), ATCC Accession No. HB-12606 (PA9), ATCC Accession No. HB-12607 (PA10), ATCC Accession No. HB-12608 (PA11), ATCC Accession No. HB-12609 (PA12) ATCC Accession No. HB-12610 (PA14).

In another embodiment of the above compositions, two or more of the compounds are antibodies. In one embodiment of the invention, the antibodies include but are not limited to PA8, PA9, PA10, PA11, PA12, PA14 and 2D7. In this composition the antibodies are in an appropriate ratio. The ratio ranges from 1:1 to 50:1.

As used herein, "antibody" means an immunoglobulin molecule comprising two heavy chains and two light

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chains and which recognizes an antigen. The immunoglobulin molecule may derive from any of the commonly known classes, including but not limited to IgA, secretory IgA, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4. It includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. Optionally, an antibody can be labeled with a detectable marker. Detectable markers include, for example, radioactive or fluorescent markers. The antibody may be a human or nonhuman antibody. The nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man. Methods for humanizing antibodies are known to those skilled in the art.

As used herein, "monoclonal antibody," also designated as mAb, is used to describe antibody molecules whose primary sequences are essentially identical and which exhibit the same antigenic specificity. Monoclonal antibodies may be produced by hybridoma, recombinant, transgenic or other techniques known to one skilled in the art.

As used herein, "anti-chemokine receptor antibody" means an antibody which recognizes and binds to an epitope on a chemokine receptor. As used herein, "anti-CCR5 antibody" means a monoclonal antibody which recognizes and binds to an epitope on the CCR5 chemokine receptor.

As used herein, "appropriate ratio" means mass or

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antibody. In one embodiment, at least one compound binds to the HIV-1 envelope glycoprotein. In one embodiment, at least one compound is an antibody to the HIV-1 envelope glycoprotein. In one embodiment, at least one compound is a CD4-based protein. In one embodiment, at least one compound is CD4-IgG2.

In another embodiment of the above compositions, at least one compound is an antibody and at least one compound binds to an HIV-1 envelope glycoprotein. In one embodiment, the compound is a CD4-based protein. In one embodiment, the compound is CD4-IgG2. In this composition, the compounds are in an appropriate ratio. The ratio ranges from 1:1 to 10:1.

As used herein, "attachment" means the process that is mediated by the binding of the HIV-1 envelope glycoprotein to the human CD4 receptor, which is not a fusion co-receptor.

As used herein, "CD4" means the mature, native, membrane-bound CD4 protein comprising a cytoplasmic domain, a hydrophobic transmembrane domain, and an extracellular domain which binds to the HIV-1 gp120 envelope glycoprotein.

As used herein, "HIV-1 envelope glycoprotein" means the HIV-1 encoded protein which comprises the gp120 surface protein, the gp41 transmembrane protein and oligomers and precursors thereof.

As used herein, "CD4-based protein" means any protein comprising at least one sequence of amino acid residues corresponding to that portion of CD4 which is required for CD4 to form a complex with the HIV-1 gp120 envelope glycoprotein.



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As used herein, "CD4-IgG2" means a heterotetrameric CD4-human IgG2 fusion protein encoded by the expression vectors deposited under ATCC Accession Numbers 75193 and 75194.

In one embodiment of the above compositions at least one of the compounds comprises a polypeptide which binds to a CCR5 epitope. In one embodiment, the epitope is located in the N-terminus, one of the three extracellular loop regions or a combination thereof. In one embodiment, the epitope is located in the N-terminus. The epitope can comprise N13 and Y15 in the N-terminus. The epitope can comprise Q4 in the N-terminus. In another embodiment, the epitope includes residues in the N-terminus and second extracellular loop. The epitope can comprise D2, Y3, Q4, S7, P8 and N13 in the N-terminus and Y176 and T177 in the second extracellular loop. The epitope can comprise D2, Y3, Q4, P8 and N13 in the N-terminus and Y176 and T177 in the second extracellular loop. The epitope can comprise D2 in the N-terminus and R168 and Y176 in the second extracellular loop. In one embodiment, the epitope is located in the second extracellular loop. The epitope can comprise Q170 and K171 in the second extracellular loop. The epitope can comprise Q170 and E172 in the second extracellular loop.

As used herein, the following standard abbreviations are used throughout the specification to indicate specific amino acids:

A=ala=alanine	R=arg=arginine
N=asn=asparagine	D=asp=aspartic acid
C=cys=cysteine	Q=gln=glutamine
E=glu=glutamic acid	G=gly=glycine
H=his=histidine	I=ile=isoleucine
L=leu=leucine	K=lys=lysine

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M=met=methionine	F=phe=phenylalanine
P=pro=proline	S=ser=serine
T=thr=threonine	W=trp=tryptophan
Y=tyr=tyrosine	V=val=valine

As used herein, "polypeptide" means two or more amino acids linked by a peptide bond.

As used herein, "epitope" means a portion of a molecule or molecules that forms a surface for binding antibodies or other compounds. The epitope may comprise contiguous or noncontiguous amino acids, carbohydrate or other nonpeptidyl moieties or oligomer-specific surfaces.

As used herein, "N-terminus" means the sequence of amino acids spanning the initiating methionine and the first transmembrane region.

As used herein, "second extra cellular loop" means the sequence of amino acids that span the fourth and fifth transmembrane regions and are presented on the surface.

In one embodiment of the above compositions at least one of the compounds comprises a light chain of an antibody. In another embodiment of the above compositions at least one of the compounds comprises a heavy chain of an antibody. In another embodiment of the above compositions at least one of the compounds comprises the Fab portion of an antibody. In another embodiment of the above compositions at least one of the compounds comprises the variable domain of an antibody. In another embodiment, the antibody is produced as a single polypeptide or "single chain" antibody which comprises the heavy and light chain variable domains genetically linked via an intervening sequence of amino acids. In another embodiment of the

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above compositions at least one of the compounds comprises one or more CDR portions of an antibody.

As used herein, "heavy chain" means the larger polypeptide of an antibody molecule composed of one variable domain (VH) and three or four constant domains (CH1, CH2, CH3, and CH4), or fragments thereof.

As used herein, "light chain" means the smaller polypeptide of an antibody molecule composed of one variable domain (VL) and one constant domain (CL), or fragments thereof.

As used herein, "Fab" means a monovalent antigen binding fragment of an immunoglobulin that consists of one light chain and part of a heavy chain. It can be obtained by brief papain digestion or by recombinant methods.

As used herein, "F(ab')<sub>2</sub> fragment" means a bivalent antigen binding fragment of an immunoglobulin that consists of both light chains and part of both heavy chains. It can be obtained by brief pepsin digestion or recombinant methods.

As used herein, "CDR" or "complementarity determining region" means a highly variable sequence of amino acids in the variable domain of an antibody.

This invention provides the above compositions and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may include but are not limited to aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and

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injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a method of treating a subject afflicted with HIV-1 which comprises administering to the subject an effective dose of the above compositions.

As used herein, "subject" means any animal or artificially modified animal capable of becoming HIV-infected. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The animals include but are not limited to mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human.

As used herein, "treating" means either slowing, stopping or reversing the progression of an HIV-1 disorder. In the preferred embodiment, "treating" means reversing the progression to the point of eliminating the disorder. As used herein, "treating" also means the reduction of the number of viral infections, reduction of the number of infectious viral particles, reduction of the number of virally infected cells, or the amelioration of symptoms associated with HIV-1.

As used herein, "afflicted with HIV-1" means that the subject has at least one cell which has been infected by HIV-1.

As used herein, "administering" may be effected or performed using any of the methods known to one skilled in the art. The methods may comprise intravenous, intramuscular or subcutaneous means.

The dose of the composition of the invention will vary depending on the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000 µg/kg. Based upon the composition, the dose can be delivered continuously, such as by continuous pump, or at periodic intervals. For example, on one or more separate occasions. Desired time intervals of multiple doses of a particular composition can be determined without undue experimentation by one skilled in the art.

As used herein, "effective dose" means an amount in sufficient quantities to either treat the subject or prevent the subject from becoming HIV-1 infected. A person of ordinary skill in the art can perform simple titration experiments to determine what amount is required to treat the subject.

This invention provides a method of preventing a subject from contracting HIV-1 which comprises administering to the subject an effective dose of the above compositions.

As used herein, "contracting HIV-1" means becoming infected with HIV-1, whose genetic information replicates in and/or incorporates into the host cells.

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This invention provides an anti-CCR5 monoclonal antibody. The antibody includes but is not limited to the following: PA8 (ATCC Accession No. HB-12605), PA9 (ATCC Accession No. HB-12606), PA10 (ATCC Accession No. HB-12607), PA11 (ATCC Accession No. HB-12608), PA12 (ATCC Accession No. HB-12609), and PA14 (ATCC Accession No. HB-12610).

This invention provides humanized forms of the above antibodies.

As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody would retain a similar antigenic specificity as the original antibody, i.e., in the present invention, the ability to bind CCR5.

One skilled in the art would know how to make the humanized antibodies of the subject invention. Various publications, several of which are hereby incorporated by reference into this application, also describe how to make humanized antibodies. For example, the methods described in United States Patent No. 4,816,567 (71) comprise the production of chimeric antibodies having a

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variable region of one antibody and a constant region of another antibody.

United States Patent No. 5,225,539 (72) describes another approach for the production of a humanized antibody. This patent describes the use of recombinant DNA technology to produce a humanized antibody wherein the CDRs of a variable region of one immunoglobulin are replaced with the CDRs from an immunoglobulin with a different specificity such that the humanized antibody would recognize the desired target but would not be recognized in a significant way by the human subject's immune system. Specifically, site directed mutagenesis is used to graft the CDRs onto the framework.

Other approaches for humanizing an antibody are described in United States Patent Nos. 5,585,089 (73) and 5,693,761 (74) and WO 90/07861 which describe methods for producing humanized immunoglobulins. These have one or more CDRs and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. These patents describe a method to increase the affinity of an antibody for the desired antigen. Some amino acids in the framework are chosen to be the same as the amino acids at those positions in the donor rather than in the acceptor. Specifically, these patents describe the preparation of a humanized antibody that binds to a receptor by combining the CDRs of a mouse monoclonal antibody with human immunoglobulin framework and constant regions. Human framework regions can be chosen to maximize homology with the mouse sequence. A computer model can be used to identify amino acids in the framework region which are likely to interact with the CDRs or the specific antigen and then mouse amino acids can be used at these positions to create the humanized antibody.

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The above patents 5,585,089 and 5,693,761, and WO 90/07861 (75) also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid residue at the framework positions at which the amino acid is predicted to have a side chain atom within 3Å of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies. The affinity and/or specificity of the binding of the humanized antibody may be increased using methods of directed evolution as described in Wu et al. (1999) J. Mol. Biol. 284:151 and U.S. Patents Nos. 6,165,793; 6,365,408 and 6,413,774.

In an embodiment of the invention the humanized form of the antibody comprises a light chain variable amino acid sequence as set forth in SEQ ID NO:6. In another embodiment, the antibody comprises a heavy chain variable amino acid sequence as set forth in SEQ ID NO:9. In a further embodiment, the antibody may comprise the heavy chain variable amino acid sequence



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as set forth in SEQ ID NO:12.

In another embodiment, the humanized antibody comprises the light chain variable amino acid sequence as set forth in SEQ ID NO:6, and the heavy chain variable amino acid sequence as set forth in SEQ ID NO:9. Alternatively, the antibody may comprise the light chain variable amino acid sequence as set forth in SEQ ID NO:6 and the heavy chain variable amino acid sequence as set forth in SEQ ID NO:12.

The variable regions of the humanized antibody may be linked to at least a portion of an immunoglobulin constant region of a human immunoglobulin. In one embodiment, the humanized antibody contains both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, CH3 and sometimes, CH4 region. In one embodiment, the constant regions of the humanized antibody are of the human IgG4 isotype.

This invention provides isolated nucleic acid molecules encoding these anti-CCR5 monoclonal antibodies or their humanized versions. The nucleic acid molecule can be RNA, DNA or cDNA. In one embodiment, the nucleic acid molecule encodes the light chain. In one embodiment, the nucleic acid molecule encodes the heavy chain. In one embodiment, the nucleic acid encodes both the heavy and light chains. In one embodiment, one or more nucleic acid molecules encode the Fab portion. In one embodiment, one or more nucleic acid molecules encode CDR portions. In one embodiment, the nucleic acid molecule encodes the variable domain. In another embodiment, the nucleic acid molecule encodes the variable domain and one or more constant domains.

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Preferably, analogs of exemplified humanized anti-CCR5 antibodies differ from exemplified humanized anti-CCR5 antibodies by conservative amino acid substitutions. For purposes of classifying amino acid substitutions as conservative or non-conservative, amino acids may be grouped as follows: Group I (hydrophobic side chains): met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

Analogues of humanized anti-CCR5 antibodies show substantial amino acid sequence identity with humanized PRO 140 #1 or humanized PRO 140 #2, exemplified herein. Heavy and light chain variable regions of analogues are encoded by nucleic acid sequences that hybridize with the nucleic acids encoding the heavy or light chain variable regions of humanized PRO 140 #1, or humanized PRO 140 #2, or degenerate forms thereof, under stringent conditions.

Due to the degeneracy of the genetic code, a variety of nucleic acid sequences encode the humanized anti-CCR5 antibody of the present invention. In certain embodiments, the antibody is encoded by a nucleic acid molecule that is highly homologous to the foregoing nucleic acid molecules. Preferably the homologous nucleic acid molecule comprises a nucleotide sequence that is at least about 90% identical to the nucleotide sequence provided herein. More preferably, the nucleotide sequence is at least about 95% identical, at

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least about 97% identical, at least about 98% identical, or at least about 99% identical to the nucleotide sequence provided herein. The homology can be calculated using various, publicly available software tools well known to one of ordinary skill in the art. Exemplary tools include the BLAST system available from the website of the National Center for Biotechnology Information (NCBI) at the National Institutes of Health.

One method of identifying highly homologous nucleotide sequences is via nucleic acid hybridization. Thus the invention also includes humanized CCR5 antibodies having the CCR5-binding properties and other functional properties described herein, which are encoded by nucleic acid molecules that hybridize under high stringency conditions to the foregoing nucleic acid molecules. Identification of related sequences can also be achieved using polymerase chain reaction (PCR) and other amplification techniques suitable for cloning related nucleic acid sequences. Preferably, PCR primers are selected to amplify portions of a nucleic acid sequence of interest, such as a CDR.

The term "high stringency conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references that compile such methods, e.g., Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. One example of high stringency conditions is hybridization at 65 degrees Centigrade in hybridization buffer (3.5X SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7), 0.5% SDS, 2mM

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EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, a membrane upon which the nucleic acid is transferred is washed, for example, in 2X SSC at room temperature and then at 0.1-0.5X SSC/0.1X SDS at temperatures up to 68 degrees Centigrade.

The nucleic acid sequences are expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms, either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent No. 4,704,362 which is incorporated herein by reference).

*E. coli* is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (*trp*) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription

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least about 97% identical, at least about 98% identical, or at least about 99% identical to the nucleotide sequence provided herein. The homology can be calculated using various, publicly available software tools well known to one of ordinary skill in the art. Exemplary tools include the BLAST system available from the website of the National Center for Biotechnology Information (NCBI) at the National Institutes of Health.

One method of identifying highly homologous nucleotide sequences is via nucleic acid hybridization. Thus the invention also includes humanized CCR5 antibodies having the CCR5-binding properties and other functional properties described herein, which are encoded by nucleic acid molecules that hybridize under high stringency conditions to the foregoing nucleic acid molecules. Identification of related sequences can also be achieved using polymerase chain reaction (PCR) and other amplification techniques suitable for cloning related nucleic acid sequences. Preferably, PCR primers are selected to amplify portions of a nucleic acid sequence of interest, such as a CDR.

The term "high stringency conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references that compile such methods, e.g., Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. One example of high stringency conditions is hybridization at 65 degrees Centigrade in hybridization buffer (3.5X SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM  $\text{NaH}_2\text{PO}_4$  (pH 7), 0.5% SDS, 2mM

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EDTA). SSC is 0.15M sodium chloride/0.015M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, a membrane upon which the nucleic acid is transferred is washed, for example, in 2X SSC at room temperature and then at 0.1-0.5X SSC/0.1X SDS at temperatures up to 68 degrees Centigrade.

The nucleic acid sequences are expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms, either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent No. 4,704,362 which is incorporated herein by reference).

*E. coli* is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (*trp*) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription

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and translation.

Other microbes, such as yeast, may also be useful for expression. *Saccharomyces* is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, New York, New York (1987)). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, preferably myeloma cell lines, etc. and transformed B cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, et al., Immunol. Rev., 89, 49-68 (1986) which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, cytomegalovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example,

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calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts (see generally, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (1982) which is incorporated herein by reference).

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see generally, R. Scopes, "Protein Purification", Springer-Verlag, New York (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like (see generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, New York (1979 and 1981)).

For diagnostic or detection purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the humanized antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels can be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands



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(particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art for detection of CCR5-expressing cells or detection of CCR5 modulation on cells capable of expressing CCR5.

The present invention also provides antibody fragment-polymer conjugates having an effective size or molecular weight that confers an increase in serum half-life, an increase in mean residence time in circulation (MRT) and/or a decrease in serum clearance rate over underivatized antibody fragments.

The antibody fragment-polymer conjugates of the invention can be made by derivatizing the desired antibody fragment with an inert polymer. It will be appreciated that any inert polymer which provides the conjugate with the desired apparent size or which has the selected actual molecular weight is suitable for use in constructing the antibody fragment-polymer conjugates of the invention.

Many inert polymers are suitable for use in pharmaceuticals. See, e.g., Davis et al., Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, pp. 441-451 (1980). In all embodiments of the invention, a non-proteinaceous polymer is used. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or in vitro methods are also useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g., polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyoxyalkylenes such as polyoxyethylene,

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polyoxypropylene and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g., polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextran sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g., hyaluronic acid, polymers of sugar alcohols such as polysorbitol and polymannitol, heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble but the final conjugate must be water soluble. Preferably, the conjugate exhibits a water solubility of at least about 0.01 mg/ml and more preferably at least about 0.1 mg/ml, and still more preferably at least about 1 mg/ml. In addition the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if the conjugate is intended to be administered by such routes.

In one embodiment, the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However it is within the scope of the invention to maximize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or ion-exchange chromatography to recover substantially homogeneous derivatives. In other embodiments the polymer contains two or more reactive groups for the purpose of linking multiple antibody fragments to the polymer backbone.

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Again, gel filtration or ion-exchange chromatography can be used to recover the desired derivative in substantially homogeneous form.

The molecular weight of the polymer can range up to about 500,000 D and preferably is at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. The molecular weight chosen can depend upon the effective size of the conjugate to be achieved, the nature (e.g., structure such as linear or branched) of the polymer and the degree of derivitization, i.e., the number of polymer molecules per antibody fragment, and the polymer attachment site or sites on the antibody fragment.

The polymer can be covalently linked to the antibody fragment through a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid residues of the antibody fragment to be linked. However, it is also within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the antibody fragment, or vice versa.

The covalent crosslinking site on the antibody fragment includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the antibody fragment without the use of a multifunctional (ordinarily bifunctional) crosslinking agent, as described in U.S. Patent No. 6,458,355.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the antibody fragment, the molecular weight, hydrophilicity

and other characteristics of the polymer, and the particular antibody fragment derivitization sites chosen. In general, the conjugate contains from 1 to about 10 polymer molecules, but greater numbers of polymer molecules attached to the antibody fragments of the invention are also contemplated. The desired amount of derivitization is easily achieved by using an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the level of polymer substitution of the conjugates is determined by size exclusion chromatography or other means known in the art.

Functionalized PEG polymers to modify the antibody fragments of the invention are available from Shearwater Polymers, Inc. (Huntsville, Ala.). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG-vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability

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for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer. The conjugates of this invention are separated from the unreacted starting materials by gel filtration or ion exchange HPLC.

The anti-CCR5 antibody or fragments thereof may be used in combination with one or more additional anti-viral agents selected from the group consisting of nonnucleoside reverse transcriptase inhibitors (NNRTIs), a nucleoside reverse transcriptase inhibitor, an HIV-1 protease inhibitor, a viral entry inhibitor and combinations thereof.

The known NNRTI compounds that may be used in the composition of the present invention include but are not limited to efavirenz, UC-781, HBY 097, nevirapine (11-cyclopropyl-5,11,-dihydro-4-methyl-6H-dipyrido[3,2-b:2'3'-][1,4] diazepin-6-one), delavirdine ((Rescriptor<sup>TM</sup>; Pharmacia Upjohn) (piperazine, 1-[3-[(1-methyl-ethyl)amino]-2-pyridinyl]-4-[[5-[(methanesulfonyl)amino]-1H-indol-2-yl]carbonyl]-, monomethanesulfonate), SJ-3366 (1-(3-cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione), MKC-442 (6-benzyl-1-(ethoxymethyl)-5-isopropyluracil), GW420867x (S-3 ethyl-6-fluoro-4-isopropoxycarbonyl-3,4-dihydro-quinoxalin-2(1H)-one; Glaxo), HI-443 (N'-[2-(2-thiophene)ethyl]-N'-[2-(5-bromopyridyl)]-thiourea), and the like.

The nucleoside reverse transcriptase inhibitors that may be used in the composition in combination with at least one anti-CCR5 antibody or fragment thereof of the present invention include but are not limited to abacavir (Ziagen<sup>TM</sup>, GlaxoSmithKline) ((1S,cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol sulfate (salt)), lamivudine

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(Epivir™, GlaxoSmithKline) ((2R, cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one), zidovudine (Retrovir™; GlaxoSmithKline) (3'-azido-3'-deoxythymidine), stavudine (Zerit; Bristol-Myers Squibb) (2',3'-didehydro-3'-deoxythymidine), zalcitabine (Hivid™; Roche Laboratories) (4-amino-1-beta-D2',3'-dideoxyribofuranosyl-2-(1H)-pyrimidone), didanosine, and the like.

The HIV-1 protease inhibitors that may be used in the composition in combination with anti-CCR5 antibody or fragments thereof of the present invention include but are not limited to lopinavir (1S-[1R\*, (R\*), 3R\*, 4R\*]]-N-4-[[[(2,6-dimethoxyphenoxy)acetyl]amino]-3-hydroxy-5-phenyl-1-(phenylmethyl)pentyl]tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H)-pyrimidineacetamide), saquinavir (N-tert-butyl-decahydro-2-[2(R)-hydroxy-4-phenyl-3(S)-[N-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl)-(4aS,8aS)-isoquinoline-(3S)-carboxamide), nelfinavir mesylate ([3S-[2(2S\*,3S\*), 3a,4β,8aβ]]-N-(1,1-dimethylethyl)decahydro-2[2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-(phenylthio)butyl]-3-isoquinolinecarboxamide monomethane sulfonate), indinavir sulfate ([1(1S,2R),5(S)]]-2,3,5-trideoxy-N-(2,3-dihydro-2-hydroxy-1H-inden-1-yl)-5-[2-[[[(1,1-dimethylethyl)amino]carbonyl]-4-(3-pyridinylmethyl)-1-piperazinyl]-2-(phenylmethyl)-D-erythropentonamide sulfate (1:1) salt], amprenavir ((3S)-tetrahydro-3-furyl N-[(1S,2R)-3-(4-amino-N-isobutylbenzenesulfonamido)-1-benzyl-2-hydroxypropyl]carbamate), ritonavir ((10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis (phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid,5-thiazolylmethyl ester, [5S-(5R\*, 8R\*, 10R\*, 11R\*)]), and the like.

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HIV-1 fusion or viral entry inhibitors that may be used in combination with the anti-CCR5 antibody or fragments thereof of the present invention include PRO 542 (Progenics Pharmaceuticals, Inc., Tarrytown, NY), T-20 (Trimeris, Inc., Durham, NC) (US Patent Nos 5,464,933; 6,133,418; 6,020,459), T-1249 (US Patent No 6,345,568; 6,258,782), and the like.

For combination therapy, the anti-CCR5 antibody or fragment thereof of the present invention may be provided to the subject prior to, subsequent to, or concurrently with one or more conventional antiviral agents.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

#### Experimental Details:

##### Example 1

#### A. Materials and Methods

##### 1) Reagents

MAb 2D7 was purchased from Pharmingen (San Diego, CA) and CC- and CXC-chemokines were obtained from R&D Systems (Minneapolis, MN). CD4-IgG2 (1), soluble (s) CD4 (2) and recombinant HIV-1<sub>JR-FL</sub> gp120, were produced by Progenics Pharmaceuticals, Inc. (59).

##### 2) Isolation and purification of anti-CCR5 mAbs

L1.2-CCR5<sup>+</sup> cells (63) were incubated for 16h in the presence of 5mM sodium butyrate, which activates

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transcription from the cytomegalovirus (CMV) promoter that controls CCR5 expression, resulting in a 10-fold increase in cell surface co-receptor density. Female Balb/c mice were immunized intraperitoneally with  $10^7$  L1.2-CCR5<sup>+</sup> cells at 3-week intervals, and administered an intravenous boost of  $10^7$  L1.2-CCR5<sup>+</sup> cells three days prior to splenectomy. Splenocytes were fused with the Sp2/0 cell line. In a primary screen, supernatants from ten thousand hybridoma cultures were tested; one hundred and twenty of these inhibited HIV-1 envelope-mediated fusion between PM1 cells (10), which naturally express CCR5 and CD4, and HeLa-Env<sub>JR-FL</sub> cells in a resonance energy transfer (RET) assay, as previously described (19, 38). Hybridomas that produced the most potently inhibitory supernatants and that also stained CCR5<sup>+</sup> cells were sub-cloned by limiting dilution. Ascites fluids were prepared by Harlan Bioproducts for Science, Inc. (Indianapolis, IN) from Balb/c mice that were injected with hybridomas producing the anti-CCR5 mAbs PA8, PA9, PA10, PA11, PA12 and PA14. The mAbs were individually purified to >95% homogeneity by precipitation with ammonium sulfate followed by protein-A chromatography. All mAbs were resuspended in phosphate buffered saline (PBS) at a final concentration of 5mg/ml.

3) Fluorescence activated cell sorting (FACS) analysis and epitope mapping of anti-CCR5 mAbs

Flow cytometry was used to detect cell-surface reactivity of mAbs PA8-PA12 and PA14 with CCR5. Sodium butyrate treated L1.2-CCR5<sup>+</sup> cells ( $10^6$ ) were incubated with 0.25 $\mu$ g of antibody, for 20min at 4°C in 0.1% sodium azide (NaN<sub>3</sub>) in 50  $\mu$ l of Dulbecco's PBS (DPBS). The CCR5 mAb 2D7 was used as a positive control, a non-specific murine IgG1 was used as a negative control. The cells were spun down, washed and incubated with phycoerythrin (PE)-labeled goat anti-mouse IgG (Caltag, Burlingame,



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CA) diluted 1:100, under the same conditions as the first antibody incubation. Finally, cells were analyzed by flow cytometry. PBMC were isolated and stimulated as previously described (60) and stained using similar methods.

A similar procedure was used for epitope mapping of the anti-CCR5 mAbs. A panel of seventy CCR5 point mutants has been described (20, 24, 52). The coding sequences of these proteins are sub-cloned into the pcDNA3.1 vector (Stratagene) from which transcription can be driven by a 5' T7-polymerase promoter. The CCR5 mutants carry a 9-residue hemagglutinin (HA) tag at the C-terminus for detection of protein in cell lysates or by flow cytometry. HeLa cells ( $2 \times 10^6$ ) were incubated for 5h with  $20 \mu\text{g/ml}$  lipofectin and an equal amount of wild-type or mutant CCR5-expressing plasmid in OPTI-MEM (Life Technologies, Gaithersburg, MD). The cells were then infected for 12h with  $2 \times 10^7$  p.f.u. of vTF7 (23) to boost CCR5 expression, detached with  $2 \text{mM}$  ethylenediamine tetracetic acid (EDTA) in PBS and washed once with binding buffer (1% BSA, 0.05% NaN<sub>3</sub> in DPBS). Cells ( $1 \times 10^6$ ) were surface labeled with mAbs as described in the previous paragraph, washed once with the incubation buffer and resuspended in  $1 \text{ml}$  of  $1 \times$  FACSlyse in water (Becton Dickinson) for 30min at room temperature, to permeabilize the cell membranes. The cells were then spun down, washed with the incubation buffer and incubated for 1h at  $37^\circ\text{C}$  with  $4 \mu\text{g/ml}$  of a fluorescein isothiocyanate (FITC)-labeled mouse anti-HA mAb (BabCo, Richmond, CA) for intracellular labeling. Finally, cells were washed once with binding buffer and once with DPBS, resuspended in 1% formaldehyde in PBS and analyzed by flow cytometry. The extent of binding of a mAb to mutant CCR5 was determined by the equation  $(\text{mutant CCR5 PE m.f.i.} / \text{wt CCR5 PE m.f.i.}) / (\text{mutant CCR5 FITC m.f.i.} / \text{wt CCR5 FITC m.f.i.}) \times 100\%$ . This

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normalizes mAb binding for mutant co-receptor expression levels.

4) gp120/sCD4-binding assay

gp120 was biotinylated using NHS-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions, and uncoupled biotin was removed by diafiltration. Sodium butyrate-treated L1.2-CCR5<sup>+</sup> cells were incubated with varying dilutions of an equimolar mixture of sCD4 and biotinylated gp120, or 1.25 µg/ml of sCD4 and 2.5 µg/ml of biotinylated gp120 in the presence of varying concentrations of anti-CCR5 mAbs PA8-PA12, PA14, 2D7 or a non-specific murine IgG1, for 1h at room temperature in 0.1% NaN<sub>3</sub> in DPBS. Cells were washed with the incubation buffer and incubated with streptavidin-PE (Becton Dickinson) diluted 1:50, for 1h at room temperature. Finally, cells were washed with binding buffer and analyzed using a fluorescence plate reader (Perspective Biosystems, Framingham, MA).

5) Inhibition of envelope-mediated cell-cell fusion and HIV-1 entry by anti-CCR5 mAbs

HIV-1 envelope-mediated fusion between HeLa-Env<sub>JR-FL</sub><sup>+</sup> and PM1 cells was detected using the RET assay. Equal numbers (2x10<sup>4</sup>) of fluorescein octadecyl ester (F18)-labeled envelope-expressing cells and octadecyl rhodamine (R18)-labeled PM1 cells were plated in 96-well plates in 15% fetal calf serum in DPBS and incubated for 4h at 37°C in the presence of varying concentrations of the anti-CCR5 mAbs, PA8-PA12, PA14, 2D7 or a non-specific murine IgG1. Fluorescence RET was measured with a Cytofluor plate-reader (PerSeptive Biosystems) and % RET was determined as previously described (38).

NLluc<sup>+</sup>env<sup>+</sup> viruses complemented in trans by envelope glycoproteins from JR-FL or Gun-1 were produced as

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previously described (20). U87MG-CD4<sup>+</sup>CCR5<sup>+</sup> cells (14) were infected with chimeric, reporter viruses containing 50-100ng/ml p24 in the presence of varying concentrations of the individual mAbs. After 2h at 37°C, virus-containing media were replaced by fresh, mAb-containing media. Fresh media, without antibodies, were added again after 12 hours. After a total of 72h, 100µl of lysis buffer (Promega) were added to the cells and luciferase activity (r.l.u.) was measured as described (20). The % inhibition of HIV-1 infection is defined as  $[1 - (\text{r.l.u. in the presence of antibody} / \text{r.l.u. in the absence of antibody})] \times 100\%$ .

#### 6) Calcium signaling assays

The fluorochrome Indo-1AM (Molecular Probes, Eugene, OR) was added to sodium butyrate treated L1.2-CCR5<sup>+</sup> cells at a final concentration of 5µM. After incubation at 37°C for 30min, the cells were washed once and resuspended in Hank's buffered saline. Cells (10<sup>6</sup>) were stimulated sequentially with an anti-CCR5 mAb or PBS, followed 60s later with RANTES. MAb PA8-PA12 and PA14 were used at a concentration of 100µg/ml, 2D7 at 20µg/ml and RANTES at 250ng/ml. Calcium flux inhibition by PA14 and 2D7 was also tested for a wide range of mAb concentrations, ranging from 0-100µg/ml. Intracellular calcium levels were monitored using a Perkin-Elmer LS-50S fluorescence spectrophotometer by measuring the ratio of fluorescence emissions at 402nm (bound dye) and 486nm (free dye) following excitation at 358nm.

### B. Results and Discussion

#### 1) Isolating anti-CCR5 monoclonal antibodies PA8, PA9, PA10, PA11, PA12 and PA14

It was found that peptides corresponding to the extracellular domains of CCR5 are inefficient at

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raising specific, high-titer antibody responses against the native, cell surface receptor (50). Balb/C mice were immunized, therefore, with L1.2-CCR5<sup>+</sup> cells and hybridoma culture supernatants were tested for their ability to inhibit JR-FL envelope-mediated membrane fusion with CD4<sup>+</sup>CCR5<sup>+</sup> PM1 cells in the RET assay (19, 38). Even though well over a hundred supernatants inhibited cell-cell fusion by >50%, only six - designated PA8, PA9, PA10, PA11, PA12 and PA14 - specifically and intensely stained L1.2-CCR5<sup>+</sup> but not the parental L1.2 cells, as demonstrated by flow cytometry (data not shown). Based on previous experience, it was assumed that the other mAbs capable of inhibiting cell-cell fusion were probably directed against cell surface adhesion molecules such as LFA-1 (37). Hybridomas PA8-PA12 and PA14 were determined by isotyping ELISA (Cappell, Durham, NC) to secrete IgG1 mAbs. Ascites fluids were prepared from Balb/C mice that were injected with the six hybridomas and the IgG1 fractions were purified. PA8, PA9, PA11, PA12 and PA14 exhibited distinct isoelectric focussing profiles, whereas PA10 had a very similar profile to that of PA9 and therefore may be a second isolate of the same mAb (data not shown).

## 2) MAb binding to CCR5<sup>+</sup> cells

None of the purified anti-CCR5 mAbs stained the parental L1.2 cell line (data not shown). However, mAbs PA9-PA12 and PA14 stained >90%, and PA8 stained ~70%, of L1.2-CCR5<sup>+</sup> cells as determined by flow cytometry, showing they recognized CCR5 (Figure 1). The anti-CCR5 mAb 2D7, which was a positive control in our experiments, also stained >90% of L1.2-CCR5<sup>+</sup> cells. PA8-PA12 and PA14 are all IgG1, and react equally well with a goat anti-mouse IgG, whereas 2D7 is an IgG2a and may react differently with the reporter antibody. Only mean fluorescence intensities (m.f.i.) measured with mAbs

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PA8-PA12 and PA14 therefore are directly comparable. The rank order of mean fluorescence intensities (m.f.i.) was PA12~ PA11> (2D7=) PA14~ PA10~ PA9> PA8. The difference between PA12 m.f.i. and PA8 m.f.i. was three-fold. Differences in staining intensity between PA8 and the other mAbs remained constant over a wide range of concentrations (data not shown) and probably do not correspond to differences in mAb affinities for CCR5. This implies that PA8 interacts only with a subset of CCR5 molecules present on the surface of L1.2-CCR5<sup>+</sup> cells.

Compared with L1.2-CCR5<sup>+</sup> cells, mitogen-stimulated PBMC exhibited different patterns of staining by the anti-CCR5 mAbs. 2D7 and PA14 stained >20%, PA11 and PA12 stained ~10%, PA8, PA9 and PA10 stained <5% of PBMC (Figure 1). The mean fluorescence intensities of the stained PBMC were about ten-fold lower than those obtained with L1.2-CCR5<sup>+</sup> cells for each mAb; their rank order was (2D7>) PA14> PA12~ PA11~ PA10~ PA9~ PA8. Again, this differed somewhat from the order of reactivities observed on CCR5 transfectants. The difference between PA9 m.f.i. and PA14 m.f.i. was seven-fold. Other groups have observed similar differences in the ability of anti-CCR5 mAbs to stain stable, CCR5<sup>+</sup> cell lines versus PBMC (28). This may be due to cell-specific differences in CCR5 conformation, post-translational modification or oligomerization. Alternatively, association with other cell surface molecules may differ between cells. Since an obvious choice for such a molecule would be the CD4 cell surface antigen, which is absent from L1.2-CCR5<sup>+</sup> cells and present on PBMCs, we also tested the ability PA8-PA12, PA14 and 2D7 to stain HeLa cells transiently expressing CCR5 alone or with CD4. No differences were observed in the ability of any of the mAbs to stain cell surface CCR5 in the presence of CD4 (data not

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shown). If there is an association between these two proteins, it does not involve epitopes recognized by the anti-CCR5 mAbs available to us. Alternatively, an association between CCR5 and CD4 might only occur on primary lymphocytes.

3) Epitope mapping of the mAbs using CCR5 alanine mutants

None of the antibodies were able to detect reduced and denatured CCR5 protein by Western blotting indicating that they recognize conformationally sensitive epitopes (data not shown). MAb epitope mapping studies were performed using a panel of seventy alanine point mutants of residues in the Nt and ECLs of CCR5. HeLa cells were lipofected with mutant or wild type CCR5 coding sequences appended with C-terminal HA tags, and infected with vTF7 (23) to boost co-receptor expression. The cells were then incubated with the anti-CCR5 mAbs and their binding was revealed by a PE-labeled goat anti-mouse IgG. A second, intracellular stain was performed with a FITC-labeled anti-HA mAb (BabCo). This internal control allowed us to directly normalize staining by the anti-CCR5 mAbs for mutant co-receptor expression levels on the cell surface. Hence, mAb binding to each mutant is expressed as a percentage of binding to wild-type CCR5 (Figure 4).

Certain point mutations reduced the binding of all of the antibodies to CCR5 by >50%. In general, PA8-PA12 were the most affected, PA14 and 2D7 the least affected by this class of mutants, which included the cysteine pair C101A and C178A, the Nt mutants Y10A, D11A, K25A, the ECL1 mutant D95A, the ECL2 mutants K171A/E172A, Q188A, K191A/N192A, and the ECL3 mutants F263A and F264A (Fig. 1). One interpretation is that these residues are not part of the mAb epitopes per se, but that changing them to alanines causes conformational

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perturbations that have a common effect on binding of all mAbs. We assumed that if a mutation lowered binding of an individual mAb by >75%, and did not also lower binding of most of the other antibodies, the residue was probably a direct contributor to the epitope recognized by the mAb. Using these stringent guidelines, it was concluded that the seven anti-CCR5 mAbs recognize overlapping but distinct epitopes (Figure 4). MAb PA8 binding to CCR5 depended on N13 and Y15 in the Nt. MAb PA9 and PA10 required D2, Y3, Q4, P8 and N13 in the Nt, and Y176 and T177 in ECL2. MAb PA9 also required S7 in the Nt. MAb PA11 and PA12 binding depended on Q4 in the Nt. PA14 required D2 in the Nt, and R168 and Y176 in ECL2. Finally, mAb 2D7 required Q170 and K171/E172 in ECL2 in order to bind to CCR5.

4) Chemokine signaling in the presence of anti-CCR5 mAbs

Chemokine receptor-binding agents can be antagonists or, more rarely, agonists of receptor-mediated intracellular signaling. Alternatively, they could have no effect on signaling. CCR5 is able to bind three CC-chemokines, RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ , and transduce a signal that modulates cytosolic calcium levels. We therefore tested the agonist/antagonist activity of various concentrations of mAbs PA8-PA12, PA14 and 2D7. Changes in intracellular calcium concentrations,  $(Ca^{2+})_i$ , were measured in Indo-1-loaded L1.2-CCR5<sup>+</sup> cells. None of the mAbs stimulated a change in  $(Ca^{2+})_i$ , indicating that they are not agonists for CCR5. PA8-PA12 were also unable to inhibit  $Ca^{2+}$  fluxes induced by RANTES (Fig.5A and data not shown), even at concentrations as high as 100 $\mu$ g/ml, showing they are not antagonists either. These concentrations provide saturating binding of the mAbs to L1.2-CCR5<sup>+</sup> cells, as shown by flow cytometry and the gp120/CCR5 binding assay (Fig. 6D and data not shown). MABs PA14 and 2D7,

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however, blocked calcium mobilization induced by RANTES, although with different potencies (Fig. 5A, 5B). The  $IC_{50}$  for PA14 calcium influx inhibition was  $50\mu\text{g/ml}$ , which was approximately 8-fold higher than the  $IC_{50}$  for 2D7 (Fig. 5B). RANTES-, MIP-1 $\alpha$ - and MIP-1 $\beta$ -induced calcium fluxes were each inhibited by similar concentrations of PA14 (data not shown). None of the mAbs affected SDF-1-induced calcium mobilization in L1.2-CCR5<sup>+</sup> cells, which endogenously express CXCR4 (data not shown). Finally, neither mAbs nor CC-chemokines affected cytosolic calcium levels in parental L1.2 cells (data not shown).

5) Inhibition of CCR5 co-receptor function by the mAbs

MAbs PA8-PA12 and PA14 were initially selected on the basis of their ability to inhibit HIV-1 envelope-mediated cell-cell fusion. This activity was confirmed and quantified for the purified mAbs. As expected, all six mAbs, as well as mAb 2D7, blocked fusion between CD4<sup>+</sup>CCR5<sup>+</sup> PM1 cells and HeLa-Env<sub>JR-FL</sub> cells in the RET assay. The rank order of potency was 2D7~ PA14> PA12> PA11> PA10~ PA9~ PA8 (Fig. 6A).  $IC_{50}$  values for PA14 and 2D7 were  $1.7\mu\text{g/ml}$  and  $1.6\mu\text{g/ml}$  respectively, for PA11 and PA12 these were  $25.5\mu\text{g/ml}$  and  $10.0\mu\text{g/ml}$  respectively (Figure 3). PA8, PA9 and PA10 inhibited fusion by only 10-15% at  $300\mu\text{g/ml}$ . None of the mAbs affected fusion between PM1 cells and HeLa-Env<sub>LAI</sub> cells, which express the full length envelope protein from an X4 virus (data not shown).

The ability of the different anti-CCR5 mAbs to inhibit entry of a prototypic R5 virus, JR-FL, and a R5X4 virus, Gun-1, in a single-round of replication, luciferase-based entry assay was also tested. The rank order of potency in the entry assay was similar to the



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one determined in the cell-cell fusion assay (Fig. 6B). A >50% inhibition of JR-FL or Gun-1 entry with PA8-PA11 was unable to be obtained. The  $IC_{50}$  value for PA12 was 2.5  $\mu\text{g/ml}$ . However, inhibition of entry by >60% with this mAb was unable to be obtained. The  $IC_{50}$  values for PA14 and 2D7 inhibition of JR-FL entry were determined to be 0.024 and 0.026  $\mu\text{g/ml}$  respectively (Figure 3), and were 60-fold lower than those obtained in the fusion assay. Entry of dual-tropic Gun-1 was 2-3-fold more sensitive to inhibition by anti-CCR5 mAbs than JR-FL entry (data not shown).

Anti-co-receptor mAbs might inhibit envelope-mediated fusion either by directly affecting the gp120/CCR5 interaction or by impeding post-binding steps involved in the formation of an active fusion complex. To determine the mechanism of inhibition of viral fusion and entry by PA8-PA12 and PA14, the ability of the different mAbs to block the gp120/CCR5 interaction was tested. For this an assay that detects binding to L1.2-CCR5<sup>+</sup> cells of biotinylated HIV-1<sub>JR-FL</sub> gp120 complexed with sCD4 was used. No binding of biotinylated gp120 was observed in the absence of sCD4 or CCR5, or when HIV-1<sub>LAI</sub> gp120 was used (Fig. 6C).

With the exception of PA8, all mAbs abrogated gp120/sCD4 binding to L1.2-CCR5<sup>+</sup> (Fig. 6D). Inhibition by PA8 saturated at ~40%, which concurs with flow cytometry data (Figure 1) in suggesting that this mAb binds only to a subset of CCR5 molecules on L1.2-CCR5<sup>+</sup> cells. Mabs PA9, PA10, PA11 and PA12 inhibited binding with  $IC_{50}$  values of 0.24, 0.13, 0.33, 0.24  $\mu\text{g/ml}$  respectively (Figure 3). Surprisingly, mAbs PA14 and 2D7 were the two least efficient inhibitors of gp120/sCD4 binding, with  $IC_{50}$  values of 1.58 and 1.38  $\mu\text{g/ml}$  respectively (Figure 3). Therefore, there was no correlation between the ability of a mAb to inhibit

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gp120/CD4/CCR5-mediated membrane fusion and entry and its ability to block gp120/sCD4 binding to the co-receptor.

6) Synergistic inhibition of HIV-1 fusion by combinations of anti-CCR5 mAbs and other viral entry inhibitors

Co-receptor-specific agents may act at multiple stages of the entry process and exhibit non-additive effects when used in combination. From a clinical perspective, it is important to determine the interactions of co-receptor-specific drug candidates with endogenous chemokines, which may afford some level of protection against disease progression. CCR5 mAbs were therefore tested in combination with each other or with RANTES, or with CD4-IgG2, which binds to HIV-1 gp120 to inhibit attachment to target cells. Dose-response curves were obtained for the agents used individually and in combination in viral fusion and entry assays. Data were analyzed using the median effect principle (9). The concentrations of single-agents or their mixtures required to produce a given effect were quantitatively compared in a term known as the Combination Index (CI). A CI value greater than 1 indicates antagonism, CI ~ 1 indicates an additive effect, and CI < 1 indicates a synergistic effect wherein the presence of one agent enhances the effect of another.

Combinations of PA12 and 2D7 were the most potently synergistic, with CI values ranging between 0.02 and 0.29, depending on the ratio of the antibodies (Fig. 7 and Figure 2). The degree of synergy is known to vary with the stoichiometry of the agents. The viral entry and fusion assays were generally consistent in identifying mAb combinations that are highly synergistic, PA12 and 2D7; moderately synergistic, PA12

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and PA14; additive, PA11 and PA12; and weakly antagonistic, PA14 and 2D7. The lack of synergy between PA14 and 2D7 is not surprising given that these mAbs cross-compete for binding to CCR5<sup>+</sup> cells as determined by flow cytometry (data not shown). The observation of an additive effect of PA11 and PA12 may be an indication that these mAbs bind to slightly different epitopes in CCR5, while sharing a dependency on residue Q4 in the Nt.

The ability of mAbs PA12, PA14 and 2D7 to synergize with RANTES in blocking cell-cell fusion was also tested. PA12 and RANTES combinations exhibited moderate synergy (Figure 2). PA14 and 2D7 exhibited no synergy with RANTES, which is consistent with these mAbs being inhibitory of RANTES binding and signaling (Fig. 5A, 5B). Finally, we tested synergy between mAbs PA12, PA14, 2D7 and CD4-IgG2, which interacts with gp120. We observed moderate synergy between PA12 and CD4-IgG2 but no synergy between PA14 or 2D7 and CD4-IgG2 (Figure 2).

#### Experimental Discussion

Six murine anti-CCR5 IgG1 mAbs were isolated and characterized. Whereas PA8, PA9, PA11, PA12 and PA14 are distinct molecular species, PA9 and PA10 are indistinguishable by the analyses and therefore are probably the same mAb. All of the mAbs that were isolated recognize complex conformational epitopes, as is often the case with mAbs raised against native, cell surface proteins. Epitope mapping was performed for all mAbs using a panel of CCR5 alanine point mutants. Residues that affected binding of all mAbs similarly were assumed to cause conformational perturbations in the co-receptor and not to constitute part of the mAb epitopes. Only two such residues, Y10 and D11, have been shown to affect HIV-1 entry (20, 52). The PA8, PA11 and PA12 epitopes are located exclusively in the

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Nt domain. Consistent with this result, PA8 was able to bind a biotinylated Nt peptide, containing residues D2 through R31, in an ELISA (data not shown). However, PA11 and PA12, whose binding strongly depended only on Q4, did not bind the Nt peptide in solution (data not shown). One possibility is that the Nt peptide does not assume the proper conformation for recognition by PA11 and PA12, whereas PA8 binding may be less conformation-dependent. Alternatively, PA11 and PA12 might interact with residues that we have not mutated, or form weak bonds with amino acids located in other domains of CCR5, or bind peptide backbone atoms whose presentation may be unchanged by mutagenesis. Antibodies PA9, PA10 and PA14 recognized epitopes that included residues in both the Nt and ECL2 domains of CCR5, whereas the 2D7 epitope was located exclusively in ECL2.

The PA14 epitope comprises both D2 in the Nt and R168 in ECL2 indicating that these two residues are proximal to one another within the context of a mAb footprint. They may even directly interact with one another through their opposite charges.

MAbs PA8-PA12 and PA14 stained CCR5<sup>+</sup> cells with different intensities and in a cell type-dependent manner. All mAbs except PA8 stained >90% L1.2-CCR5<sup>+</sup> cells, the highest mean fluorescence intensity being observed with PA11 and PA12. However, PA14 and 2D7 stained the highest percentage of PBMC and also yielded the highest mean fluorescence intensities on these cells. Hill et al. (28) have recently characterized a panel of anti-CCR5 mAbs that similarly stained transfected cells, but only two of eight stained PBMC, and none stained primary monocytes. A low affinity for CCR5 probably accounted for the non-reactivity of two of the mAbs with primary cells, but this was unlikely to be the explanation for the failure of the other four

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to react. In our mAb panel, we observe the most intense staining of PBMC by mAbs 2D7 and PA14 that have epitopes located entirely or partially in the first ten residues of ECL2. Hill et al. report, however, that mAbs specific for the Nt and ECL1 stain PBMCs, while mAbs to ECL2 and ECL3 do not stain PBMC, so a consistent pattern of reactivity has not been identified. One explanation for cell type-specific staining by mAbs would be that activated PBMCs (and monocytes) secrete CC-chemokines that bind to cell surface CCR5, masking some mAb epitopes. However, one would expect this to be especially true for PA14 and 2D7, which are antagonists of chemokine-induced calcium mobilization and presumably compete with CC-chemokines for binding to CCR5. Yet these mAbs stain PBMC the most intensely. Alternatively, differential CCR5 epitope exposure may reflect cell type-specific receptor oligomerization, association with other cell-surface molecules, or different post-translational modifications such as glycosylation. We have shown that differences in mAb binding probably do not reflect cell type-specific differences in CD4/CCR5 interactions.

MAbs PA8-PA12 did not inhibit CC-chemokine induced calcium mobilization in CCR5<sup>+</sup> cells, nor did they mediate signaling through CCR5. MAbs 2D7 and PA14 were inhibitors of CC-chemokine induced calcium mobilization, but 2D7 was almost an order of magnitude more potent than PA14. This may be because the PA14 epitope overlaps less with the CC-chemokine binding domain on CCR5 than the 2D7 epitope. All of the mAbs also blocked HIV-1 entry and envelope-mediated membrane fusion, but inhibition of cell-cell fusion required in some cases almost two orders of magnitude more antibody than what was needed to block viral entry. Presumably, more gp120/CD4/CCR5 interactions as well as interactions between adhesion molecules are established

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and act cooperatively during cell-cell fusion, compared to virus-cell fusion, making it more difficult to inhibit. This is commonly observed with antibodies to LFA-1 or to the HIV-1 envelope glycoprotein (45, 51). PA8, PA9 and PA10 were unable to block cell-cell fusion by >15% and viral entry by >40%, even at the highest antibody concentrations. However, >90% inhibition of fusion could be attained with PA11, PA12 and PA14, and >90% inhibition of entry could be attained with PA14. The most potent of the six mAbs in blocking fusion and entry was PA14, which was as effective as 2D7. Surprisingly, PA14 and 2D7 were among the least potent inhibitors of gp120/sCD4 binding to L1.2-CCR5<sup>+</sup> cells, whereas PA9-PA12 blocked with similar potencies, and PA8 was unable to block >40% of gp120/sCD4 binding. These observations raise questions about the nature of the CCR5 molecules presented on different cells and about the mechanisms of inhibition of viral fusion and entry. It may be that CCR5 on L1.2 cells, used in the mAb and gp120-binding assays, is not in an identical conformation to CCR5 on PBMC, used in the mAb-binding assay, or to CCR5 on PM1 and U87MG cells used in the fusion and entry assays.

The low staining of PBMC and the partial inhibition of fusion and entry by some of our mAbs indicate that they are only able to bind to a subset of CCR5 molecules expressed on primary lymphocytes, PM1 and U87MG-CD4<sup>+</sup>CCR5<sup>+</sup> cell lines. Yet, other than PA8, all mAbs are able to stain >90% L1.2-CCR5<sup>+</sup> cells and to completely block binding of the gp120/sCD4 complex to these cells. At least one difference between L1.2-CCR5<sup>+</sup> and the other cells that we have used is the density of co-receptor protein on the cell surface. Indeed, we estimate that the L1.2-CCR5<sup>+</sup> cells express 10- to 100-fold more cell surface co-receptor than PM1 and U87MG-CD4<sup>+</sup>CCR5<sup>+</sup> cells. But when HeLa cells are engineered to transiently

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express as much co-receptor as the L1.2-CCR5<sup>+</sup> cell line, we are still unable to detect gp120/sCD4 binding to them (data not shown). Over-expression of CCR5 on L1.2, along with other cell-specific factors therefore, might favor a co-receptor conformation that prominently exposes the Nt, making it more accessible to both mAbs and gp120. Such a conformation might be induced by receptor oligomerization, by diminished or altered associations with cell surface proteins or by receptor interactions with G proteins (25, 62). Do multiple conformations of CCR5 co-exist on the cell surface, and are they all permissive for viral entry? The patterns of mAb reactivity would suggest so, since HIV-1 entry and fusion can occur, albeit at reduced levels, in the presence of mAb concentrations that saturate epitopes required for gp120 binding to L1.2-CCR5<sup>+</sup> cells. We favor the hypothesis that the co-receptor molecules present on L1.2-CCR5<sup>+</sup> cells possess one HIV-1 entry-competent conformation whereas CCR5 molecules on PBMC, PM1 and CCR5<sup>+</sup> U87MG exist in multiple, entry-competent states that display different mAb reactivities. Whereas PA14 and 2D7 may recognize all conformations, other mAbs may not. Why L1.2 cells are conducive to a particular fusion-competent conformation remains to be determined.

It has recently been demonstrated that the gp120-binding domain lies in the first twenty residues of the CCR5 Nt domain. MAbs to the gp120-binding domain on CCR5 potentially block this interaction but are not nearly as efficient at inhibiting HIV-1 fusion and entry into target cells as PA14 and 2D7, whose epitopes lie outside this region. PA14 recognizes the tip of the Nt and residues in ECL2, whereas the 2D7 epitope is located exclusively in ECL2. At the mechanism of action of these mAbs can only be speculated. It may be that their binding to the first few residues of ECL2 induces

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conformational changes in the co-receptor that prevent membrane fusion. Alternatively, obstruction of ECL2 epitopes might impede co-receptor oligomerization and the formation of a fusion-competent protein complex. Yet another possibility is that residues in ECL2 face the inside of the fusion pore and binding of the mAbs impedes gp41 from inserting the fusion peptide into the plasma membrane. In contrast, mAbs PA8-PA12 probably inhibit fusion and entry only by directly competing for binding with gp120/CD4 complexes. We do not know if parameters other than epitope exposure and affinity for CCR5 determine the efficacy of viral entry inhibition by these mAbs. It is unclear why inhibiting steps subsequent to the gp120/co-receptor interaction would be more efficient than directly blocking that interaction. One way to explain this would be to assume that the off rate of gp120 binding to CCR5 is much lower than the on rate of mAb binding to CCR5. Thus, every time a mAb detaches itself from a co-receptor molecule, a virion-associated gp120 molecule replaces it in a quasi-irreversible fashion since this interaction leads to membrane fusion.

Synergy between combinations of anti-CCR5 mAbs is probably a result of their interactions with distinct epitopes that are involved in inter-dependent, consecutive steps of HIV-1 entry. The degree of synergy observed between PA12 and 2D7 ( $CI < 0.1$  under many circumstances) is extraordinary since  $CI$  values  $< 0.2$  are rarely observed for combinations of anti-HIV-1 antibodies (33, 35, 61), reverse transcriptase inhibitors (29), or protease inhibitors (44). Because of its potency, the PA12:2D7 combination was examined in multiple assay formats and concentration ratios, for which consistently high levels of synergy were observed. Moderate synergy was observed for PA12 combined with PA14. We also observed moderate synergy



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between PA12 and CD4-IgG2. The CD4/gp120 complex is metastable and if it is unable to interact with a co-receptor, decays into a non-fusogenic state (45-48). Since PA12 directly blocks the gp120-binding site on CCR5, its presence may shift the equilibrium towards inactivation of the gp120/CD4 complex. This would explain why we observe synergy between CD4-IgG2 and mAb PA12 with respect to inhibition of fusion and entry. The lack of synergy between mAb PA14 and CD4-IgG2 suggests that they act on two non-consecutive and independent steps of viral entry. A combination of further studies will be needed to determine the precise mechanisms of synergy of the different compounds with respect to inhibition of viral fusion and entry.

The above results are consistent with a model wherein HIV-1 entry occurs in three distinct steps involving receptor binding, co-receptor binding, and co-receptor mediated membrane fusion. Separate co-receptor binding and fusion events are suggested by the lack of correlation between the monoclonal antibodies' abilities to block gp120 binding and HIV-1 fusion/entry. The chronology of events during fusion is further suggested by the patterns of synergies observed. Agents, such as PA12, that potently inhibit the middle step of the process, namely gp 120 binding, act synergistically with inhibitors of prior and subsequent steps.

#### Example 2

**Background:** The increasing incidence of multidrug-resistant HIV-1 mandates the search for novel classes of antiretroviral agents. CCR5 is a requisite fusion coreceptor for primary HIV-1 isolates and provides a promising target for antiviral therapy. PRO140 is an

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anti-CCR5 monoclonal antibody that potently inhibits HIV-1 entry and replication at concentrations that do not affect CCR5's chemokine receptor activity *in vitro*. In the present study, we evaluated the therapeutic potential of PRO 140 *in vivo* using a therapeutic animal model of HIV-1 infection.

**Methods:** CD-17 SCID mice were reconstituted with normal human PBMC and infected with the R5 isolate HIV-1 JR-CSF. When viral steady state was reached, the animal were treated intraperitoneally with PRO 140 or control antibody and monitored for viral burden using the Roche Amplicor assay. Initial studies examined a single 1 mg dose of PRO140. In multi-dose studies, PRO 140 was administered once every three days for three weeks at doses ranging from 0.1-1.0 mg. In a separate experiment, flow cytometry was used to examine the potential for lymphocyte depletion following PRO 140 injection.

**Results:** Both single-dose and multi-dose PRO 140 reduced viral loads to undetectable levels in all treated animals, and the viral load reductions ranged to 1.8 log 10. A transitory control of viral replication was observed following single injection of PRO 140 while multiple injections led to a prolonged control with no evidence of viral rebound during therapy. Dose-dependent differences were observed in the kinetics of the PRO 140-mediated reductions in viral load. Flow cytometry analysis showed that treatment with PRO 140 did not lead to lymphocyte depletion, confirming that impact on viral replication *in vivo* was solely due to CCR5-blockage.

Conclusions: PRO 140 is highly effective in controlling established HIV-1 infection in the hu-PBL-SCID mouse model of HIV-1 infection. These findings provide in vivo proof-of-concept for PRO 140 therapy in particular and for CCR5-inhibitors therapy in general.

### Example 3

#### Methods:

A humanized CCR5 antibody (huPRO 140) was tested for the ability to block RANTES-induced calcium mobilization in L1.2-CCR5 cells and the ability to block replication of HIV-1 CASE C 1/85 in human PBMC's using methods described herein.

#### Results:

The results as shown in Figure 19 shows that the humanized CCR5 antibody potently blocks HIV-1 but not RANTES.

#### REFERENCES

1. Allaway, G.P., K.L. Davis-Bruno, B.A. Beaudry, E.B. Garcia, E.L. Wong, A.M. Ryder, K.W. Hasel, M.C. Gauduin, R.A. Koup, J.S. McDougal and P.J. Maddon. 1995. Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates. AIDS Res Hum Retroviruses 11: 533-539.
2. Allaway, G.P., A.M. Ryder, G.A. Beaudry and P.J. Maddon. 1993. Synergistic inhibition of HIV-1 envelope-mediated cell fusion by CD4-based molecules in combination with antibodies to gp120 or gp41. AIDS Res Hum Retroviruses 9: 581-587.
3. Amara, A., S.L. Gall, O. Schwartz, J. Salamero, M.

-67-

- Montes, P. Loetscher, M. Baggiolini, J.L. Virelizier and F. Arenzana-Seisdedos. 1997. HIV coreceptor downregulation as antiviral principle: SDF-1 $\alpha$ -dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J. Exp. Med.* 186: 139-146.
4. Berger, E.A. 1997. HIV entry and tropism: the chemokine receptor connection. *AIDS* 11 (suppl A): S3-S16.
  5. Bieniasz, P.D. and B.R. Cullen. 1998. Chemokine receptors and human immunodeficiency virus infection. *Frontiers in Bioscience* 3: d44-58.
  6. Bieniasz, P.D., R.A. Fridell, I. Aramori, S.S.G. Ferguson, M.C. Caron and B.R. Cullen. 1997. HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR5 co-receptor. *EMBO* 16: 2599-2609.
  7. Brelot, A., N. Heveker, O. Pleskoff, N. Sol and M. Alizon. 1997. Role of the first and third extracellular domains of CXCR4 in human immunodeficiency virus coreceptor activity. *J. Virol.* 71: 4744-4751.
  8. Chan, D.C. and P.S. Kim. 1998. HIV entry and its inhibition. *Cell* 93: 681-684.
  9. Chou, T.C. and D.C. Rideout. Synergism and antagonism in chemotherapy. New York: Academic Press, 1991
  10. Cocchi, F., A.L. DeVico, A. Garzino-Derno, S.K. Arya, R.C. Gallo and P. Lusso. 1995. Identification of RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  as the major HIV-suppressive factors produced by CD8 T-cells. *Science* 270: 1811-1815.
  11. Connor, R.I., K.E. Sheridan, D. Ceradini, S. Choe and N.R. Landau. 1997. Change in co-receptor use correlates with disease progression in HIV-1 infected individuals. *J. Exp. Med.* 185: 621-628.

-68-

12. Crump, M.P., J.H. Gong, P. Loetscher, K. Rajarathnam, A. Amara, F. Arenzana-Seisdedos, J.L. Virelizier, M. Baggiolini, B.D. Sykes and I. Clark-Lewis. 1997. Solution structure and basis for functional activity of stromal-cell derived factor-1; disassociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO* 16: 6996-7007.
13. Dalglish, A.G., P.C.L. Beverly, P.R. Clapham, D.H. Crawford, M.F. Greaves and R.A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312: 763-766.
14. Deng, H.K., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. DiMarizio, S. Marmon, R.E. Sutton, C.M. Hill, S.C. Peiper, T.J. Schall, D.R. Littman and N.R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381: 661-666.
15. Dimitrov, D.S. 1997. How do viruses enter cells? The HIV Co-receptors teach us a lesson of complexity. *Cell* 91: 721-730.
16. Donzella, G.A., D. Schols, S.W. Lin, K.A. Nagashima, P.J. Maddon, G.P. Allaway, T.P. Sakmar, E.D. Clercq and J.P. Moore. 1998. JM3100, a small molecule that interacts with the CXCR4 co-receptor to prevent HIV-1 entry. *Nat. Med.* 4: 72-77.
17. Doranz, B.J., K. Grovit-Ferbas, M.P. Sharron, S.H. Mao, M.B. Goetz, E.S. Daar, R.W. Doms and W.A. O'Brien. 1997. A small molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 co-receptor. *J. Ex. Med.* 186: 1395-1400.
18. Doranz, B.J., Z.-H. Lu, J. Rucker, T.-Y. Zhang, M. Sharron, Y.-H. Cen, Z.-X. Wang, H.-H. Guo, J.-G. Du, M.A. Accavitti, R.W. Doms and S.C. Peiper.

-69-

1997. Two distinct CCR5 domains can mediate co-receptor usage by human immunodeficiency virus type 1. *J. Virol.* 71: 6305-6314.
19. Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huanh, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P. Moore and W.A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381: 667-673.
  20. Dragic, T., A. Trkola, X.W. Lin, K.A. Nagashima, F. Kajumo, L. Zhao, W.C. Olson, L. Wu, C.R. Mackay, G.P. Allaway, T.P. Sakmar, J.P. Moore and P.J. Maddon. 1998. Amino terminal substitutions in the CCR5 co-receptor impair gp120 binding and human immunodeficiency virus type 1 entry. *J. Virol.* 72: 279-285.
  21. Dragic, T., A. Trkola and J.P. Moore. 1997. HIV co-receptors: Gateways to the cell. *Advances in Research and Therapy* 7: 2-13.
  22. Farzan, M., H. Choe, L. Vaca, K. Martin, Y. Sun, E. Desjardins, N. Ruffing, L. Wu, R. Wyatt, N. Gerard, C. Gerard and J. Sodroski. 1998. A tyrosine-rich region in the N-terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. *J. Virol.* 72: 1160-1164.
  23. Fuerst, T.R., E.G. Niles, F.W. Studier and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA.* 83: 8122-8126.
  24. Genoud, S., F. Kajumo, Y. Guo, D.A.D. Thompson and T. Dragic. CCR5-mediated human immunodeficiency virus entry depends on an amino-terminal domain gp120-binding site and on the conformational integrity of all four extracellular domains. *J. Virol.* submitted.

25. Gether, U. and B.K. Kobilka. 1998. G protein-coupled receptors. *J. Biol. Chem* **273**: 17979-17982.
26. Gordon, C., M. Muesing, A.E.I. Proudfoot, C.A. Power, J.P. Moore and A. Trkola. 1998. Enhancement of human immunodeficiency virus type 1 infection by the CC-chemokine RANTES is independent of the mechanism of virus-cell fusion. *J. Virol.* in press.
27. Heveker, N., M. Montes, L. Germeroth, A. Amara, A. Trautmann, M. Alizon and J. Schneider-Mergener. 1998. Dissociation of the signaling and antiviral properties of SDF-1-derived small peptides. *Current Biology* **8**: 369-376.
28. Hill, C.M., D. Kwon, M. Jones, C.B. Davis, S. Marmon, B.L. Daugherty, J.A. DeMartino, M.S. Springer, D. Unutmaz and D.R. Littman. 1998. The amino terminus of human CCR5 is required for its function as a receptor for diverse human and simian immunodeficiency virus envelope glycoproteins. *Virology* **248**: 357-371.
29. Johnson, V.A., D.P. Merrill, J.A. Videler, T.C. Chou, R.E. Byington, J.J. Eron, R.T. D'Aquila and M.S. Hirsch. 1991. Two-drug combinations of zidovudine, didanosine, and recombinant interferon-alpha A inhibit replication of zidovudine-resistant human immunodeficiency virus type 1 synergistically in vitro. *J Infect Dis* **164**: 646-655.
30. Klatzmann, D., E. Champagne, S. Chamaret, J.M. Gruest, D. Guetard, T. Hercend, J.C. Gluckman and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**: 382-385.
31. Kuhmann, K.E., E.J. Platt, S.L. Kozak and D. Kabat. 1997. Polymorphism in the CCR5 genes of African green monkeys and mice implicate specific

-71-

- amino acids in infections by simian and human immunodeficiency viruses. *J. Virol.* 71: 8642-8656.
32. Kwong, P.D., R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski and W.A. Hendrickson. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393: 648-659.
  33. Laal, S., S. Burda, M.K. Gorny, S. Karwowska, A. Buchbinder and S. Zolla-Pazner. 1994. Synergistic neutralization of human immunodeficiency virus type 1 by combinations of human monoclonal antibodies. *J. Virol.* 68: 4001-4008.
  34. Labrosse, B., A. Brelot, N. Heveker, N. Sol, D. Schols, E.D. Clercq and M. Alizon. 1998. Determinants for sensitivity of human immunodeficiency virus co-receptor CXCR4 to the bicyclam AMD3100. *J. Virol.* 72: 6381-6388.
  35. Li, A., H. Katinger, M.R. Posner, L. Cavacini, S. Zolla-Pazner, M.K. Gorny, J. Sodroski, T.C. Chou, T.W. Baba and R.M. Ruprecht. 1998. Synergistic neutralization of simian-human immunodeficiency virus SHIV-vpu+ by triple and quadruple combinations of human monoclonal antibodies and high-titer anti-human immunodeficiency virus type 1 immunoglobulins. *J. Virol.* 72: 3235-3240.
  36. Littman, D.R. 1998. Chemokine receptors: keys to AIDS pathogenesis. *Cell* 93: 677-680.
  37. Litwin, V. unpublished results.
  38. Litwin, V., K. Nagashima, A.M. Ryder, C.H. Chang, J.M. Carver, W.C. Olson, M. Alizon, K.W. Hasel, P.J. Maddon and G.P. Allaway. 1996. Human immunodeficiency virus type 1 membrane fusion mediated by a laboratory-adapted strain and a primary isolate analyzed by resonance energy transfer. *J. Virol.* 70: 6437-6441.
  39. Loetscher, P., J.H. Gong, B. Dewald, M. Baggiolini



-72-

- and I. Clark-Lewis. 1998. N-terminal peptides of stromal cell derived factor-1 with CXC chemokine receptor 4 agonist and antagonist activities. *J. Biol. Chem.* 273: 22279-22283.
40. Mack, M., B. Luckow, P.J. Nelson, J. Cihak, G. Simmons, P.R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, T.N.C. Wells, D. Schlondorff and A.E.I. Proudfoot. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanisms of HIV infectivity. *J. Ex. Med.* 187: 1215-1224.
41. Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* 47: 333-348.
42. McDougal, J.S., M.S. Kennedy, J.M. Sligh, S.P. Cort, A. Mawle and J.K.A. Nicholson. 1986. Binding of HTLVIII / LAV to T4<sup>+</sup> T cells by a complex of the 110K viral protein and the T4 molecule. *Science* 231: 382-385.
43. McKnight, A., D. Wilkinson, G. Simmons, S. Talbot, L. Picard, M. Ahuja, M. Marsh, J.A. Hoxie and P.R. Clapham. 1997. Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a co-receptor (CXCR4) is both cell type and virus strain dependent. *J. Virol.* 71: 1692-1696.
44. Merrill, D.P., D.J. Manion, T.C. Chou and M.S. Hirsch. 1997. Antagonism between human immunodeficiency virus type 1 protease inhibitors indinavir and saquinavir in vitro. *J Infect Dis* 176: 265-268.
45. Moore, J.P., Y. Cao, L. Qing, Q.J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C.F. Barbas, D.R.

-73-

- Burton and D.D. Ho. 1995. Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120 and their neutralization is not predicted by studies with monomeric gp120. *J. Virol.* 69: 101-109.
46. Moore, J.P., B.A. Jameson, R.A. Weiss and Q.J. Sattentau. The HIV-cell fusion reaction. Boca Raton: CRC Press Inc., 1993 (J. Bentz, ed. *Viral Fusion Mechanisms*)
47. Moore, J.P., J.A. McKeating, Y. Huang, A. Ashkenazi and D.D. Ho. 1992. Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. *J. Virol.* 66: 235-243.
48. Moore, J.P. and R.W. Sweet. 1993. The HIV gp120-CD4 interaction: a target for pharmacological and immunological intervention. *Prospect in Drug Discovery and Design* 1: 235-250.
49. Murakami, T., T. Nakajima, Y. Koyanagi, K. Tachibana, N. Fujii, H. Tamamura, N. Yoshida, M. Waki, A. Matsumoto, O. Yoshie, T. Kishimoto, N. Yamamoto and T. Nagasawa. 1997. A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J. Ex. Med.* 186: 1389-1393.
50. Olson, W.C. unpublished results.
51. Pantaleo, G., G. Poli, L. Butini, C. Fox, A.I. Dayton and A.S. Fauci. 1991. Dissociation between syncytia formation and HIV spreading. Suppression of syncytia does not necessarily reflect inhibition of HIV infection. *Eur. J. Immunol.* 21: 1771-1774.
52. Rabut, G.E.E., J.A. Konner, F. Kajumo, J.P. Moore and T. Dragic. 1998. Alanine substitutions of

-74-

- polar and non-polar residues in the amino-terminal domain of CCR5 differently impair entry of macrophage- and dual-tropic isolates of the human immunodeficiency virus type 1. *J. Virol.* 72: 3464-3468.
53. Rizzuto, C., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P. Kwong, W. Hendrickson and J. Sodroski. 1998. Identification of a conserved human immunodeficiency virus gp120 glycoprotein structure important for chemokine receptor binding. *Science* 280: 1949-1953.
54. Rucker, J., M. Samson, B.J. Doranz, F. Libert, J.P. Berson, Y. Yi, R.J. Smyth, R.G. Collman, C.C. Broder, G. Vassart, R.W. Doms and M. Parmentier. 1996. Regions in the  $\beta$ -chemokine receptors CCR-5 and CCR-2b that determine HIV-1 cofactor specificity. *Cell* 87: 437-446.
55. Schols, D., S. Struyf, J.V. Damme, J.A. Este, G. Henson and E.D. Clercq. 1997. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J. Ex. Med.* 186: 1383-1388.
56. Simmons, G., P.R. Clapham, L. Picard, R.E. Offord, M.M. Rosenkilde, T.W. Schwartz, R. Buser, T.N.C. Wells and A.E.I. Proudfoot. 1997. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 276: 276-279.
57. Simmons, G., D. Wilkinson, J.D. Reeves, M.T. Dittmar, S. Beddows, J. Weber, G. Carnegie, U. Desselberger, P.W. Gray, R.A. Weiss and P.R. Clapham. 1996. Primary, syncytium-inducing human immunodeficiency virus type-1 isolates are dual-tropic and most can use either LESTR or CCR5 as co-receptor for virus entry. *J. Virol.* 70: 8355-8360.

-75-

58. Strizki, J.M., J. Davis-Turner, R.G. Collman, J. Hoxie and F. Gonzalez-Scarano. 1997. A monoclonal antibody (12G5) directed against CXCR4 inhibits infection with the dual-tropic human immunodeficiency virus type 1 isolate HIV-1 89.6 but not the T-tropic isolate HIV-1 HxB. *J. Virol.* 71: 5678-5683.
59. Trkola, A., T. Dragic, J. Arthos, J. Binley, W.C. Olson, G.P. Allaway, C. Cheng-Mayer, J. Robinson, P.J. Maddon and J.P. Moore. 1996. CD4-dependent, antibody sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* 384: 184-187.
60. Trkola, A., W.A. Paxton, S.P. Monard, J.A. Hoxie, M.A. Siani, D.A. Thompson, L. Wu, C.R. Mackay, R. Horuk and J.P. Moore. 1997. Genetic subtype-independent inhibition of human immunodeficiency virus type-1 replication by CC- and CXC chemokines. *J. Virol.* 72: 396-404.
61. Vijh-Warrier, S., A. Pinter, W.J. Honnen and S.A. Tilley. 1996. Synergistic neutralization of human immunodeficiency virus type 1 by a chimpanzee monoclonal antibody against the V2 domain of gp120 in combination with monoclonal antibodies against the V3 loop and the CD4-binding site. *J. Virol.* 70: 4466-4473.
62. Ward, S.G., K. Bacon and J. Westwick. 1998. Chemokines and lymphocytes: more than an attraction. *Immunity* 9: 1-11.
63. Wu, L., N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A.A. Cardoso, E. Desjardin, W. Newman, C. Gerard and J. Sodroski. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 384: 179-183.
64. Wu, L., G. LaRosa, N. Kassam, C.J. Gordon, H. Heath, N. Ruffing, H. Chen, J. Humblas, M.

-76-

- Samson, M. Parmentier, J.P. Moore and C.R. Mackay. 1997. Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *J. Exp. Med.* 186: 1373-1381.
65. Wyatt, R., P.D. Kwong, E. Desjardins, R. Sweet, J. Robinson, W. Hendrickson and J. Sodroski. 1998. The antigenic structure of the human immunodeficiency virus gp120 envelope glycoprotein. *Nature* 393: 705-711.
66. Wyatt, R. and J. Sodroski. 1998. The HIV-1 envelope glycoproteins: fusogens, antigens and immunogens. *Science* 280: 1884-1888.
67. Ylisastigui, L., J.J. Vizzavona, E. Drakopoulou, P. Paindavoine, C.F. Calvo, M. Parmentier, J.C. Gluckman, C. Vita and A. Benjouad. 1998. Synthetic full length and truncated RANTES inhibit HIV-1 infection of primary macrophages. *AIDS* 12: 977-984.
68. Zhang, J.L., H. Choe, B.J. Dezube, M. Farzan, P.L. Sharma, X.C. Zhou, L.B. Chen, M. Ono, S. Gillies, Y. Wu, J.G. Sodroski and C.S. Crumpacker. 1998. The bis-azo compound FP-21399 inhibits HIV-1 replication by preventing viral entry. *Virology* 244: 530-541.
69. Cairns, J.S., D'Souza. M.P., 1998. Chemokines and HIV-1 second receptors: the therapeutic connection. *Nature Medicine*. 1998. Vol 4, No. 5: 563.
70. Kilby, J. Michael, et al. 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nature Medicine*. Vol. 3, No. 11: 1302.

-77-

71. United States Patent No. 4,816,567, issued March 28, 1989 to Cabilly et al.
72. United States Patent No. 5,225,539, issued July 6, 1993 to Gregory Winter.
73. United States Patent No. 5,585,089, issued December 17, 1996 to Queen et al.
74. United States Patent No. 5,693,761, issued December 2, 1997 to Queen et al.
75. PCT International Application No. PCT/US89/05857, filed December 28, 1989, published July 26, 1990, WO 90/07861.

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CLAIMS

What is claimed:

1. An anti-CCR5 antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell.
2. The anti-CCR5 antibody of claim 1, wherein the heavy chains are expressed by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098).
3. The anti-CCR5 antibody of claim 1, wherein the heavy chains are expressed by the plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099).
4. An anti-CCR5 antibody comprising two light chains, each chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6, and two heavy chains, each heavy chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:9.
5. An anti-CCR5 antibody comprising two light chains, each light chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6, and two heavy chains, each

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heavy chain comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:12.

6. An isolated nucleic acid encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:6.
7. The nucleic acid of claim 6, wherein the consecutive amino acids are the amino acids expressed by a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097).
8. The nucleic acid of claim 6, wherein the nucleic acid comprises the sequence set forth in SEQ ID NO:5.
9. The nucleic acid of any one of claims 6, 7 or 8, wherein the nucleic acid is RNA, DNA or cDNA.
10. An isolated nucleic acid encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:9.
11. The nucleic acid of claim 10, wherein the consecutive amino acids are the amino acids expressed by a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098).
12. The nucleic acid of claim 10, wherein the nucleic acid comprises the sequence set forth in SEQ ID NO:8.
13. The nucleic acid of any one of claims 10, 11 or 12 wherein the nucleic acid is RNA, DNA or cDNA.



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14. An isolated nucleic acid encoding a polypeptide comprising consecutive amino acids, the amino acid sequence of which is set forth in SEQ ID NO:12.
15. The nucleic acid of claim 14, wherein the consecutive amino acids are the amino acids expressed by a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099).
16. The nucleic acid of claim 14, wherein the nucleic acid comprises the sequence set forth in SEQ ID NO:11.
17. The nucleic acid of any one of claims 14, 15 and 16, wherein the nucleic acid is RNA, DNA or cDNA.
18. A composition comprising at least one of the anti-CCR5 antibody or a fragment thereof, of any one of claims 1-5 and a carrier.
19. A composition comprising the anti-CCR5 antibody or a fragment thereof, of any one of claims 1-5, having attached thereto a material selected from the group consisting of a radioisotope, a toxin, polyethylene glycol, a cytotoxic agent and a detectable label.
20. A method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with an antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation

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PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody which binds to CCR5 on the surface of the CD4+ cell, in an amount and under conditions such that fusion of HIV-1 or an HIV-1 infected cell to the CD4+ cell is inhibited, thereby inhibiting HIV-1 infection of the CD4+ cell.

21. The method of claim 20, wherein the CD4+ cell expresses CCR5.

22. A method of treating a subject afflicted with HIV-1 which comprises administering to the subject an effective HIV-1 treating dosage amount of an anti-CCR5 antibody comprising (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell, under conditions effective to treat said HIV-1-afflicted subject.

23. A method of preventing a subject from contracting an HIV-1 infection which comprises administering to the subject an effective HIV-1 infection-preventing dosage amount of an anti-CCR5 antibody comprising (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains,

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each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO 140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody, which binds to CCR5 on the surface of a human cell, under conditions effective to prevent said HIV-1 infection in said subject.

24. The method of claim 22 or 23, wherein the anti-CCR5 antibody is administered to the subject by a method selected from the group consisting of intravenous, intramuscular and subcutaneous means.
25. The method of claim 22 or 23, wherein the anti-CCR5 antibody is administered continuously to said subject.
26. The method of claim 22 or 23 wherein the anti-CCR5 antibody is administered at predetermined periodic intervals to said subject.
27. The method of claim 22 or 23, which further comprises labeling the anti-CCR5 antibody with a detectable marker.
28. The method of claim 27, wherein the detectable marker is a radioactive or a fluorescent marker.
29. The method of claim 22 or 23, wherein the dosage of said anti-CCR5 antibody ranges from about 0.1 to about 100,000  $\mu\text{g/kg}$  body weight of said subject.

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30. The method of claim 29, wherein the dosage of said anti-CCR5 antibody does not inhibit an endogenous chemokine activity on CCR5 in said subject.
31. An anti-CCR5 antibody conjugate comprising an anti-CCR5 antibody which comprises (i) two light chains, each light chain comprising the expression product of a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) two heavy chains, each heavy chain comprising the expression product of either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), or a fragment of such antibody which binds to CCR5 on the surface of a human cell, conjugated to at least one polymer.
32. The anti-CCR5 antibody conjugate of claim 31, wherein the polymer is selected from the group consisting of hydrophilic polyvinyl polymers, polyalkylene ethers, polyoxyalkylenes, polymethacrylates, carbomers, branched polysaccharides, unbranched polysaccharides, polymers of sugar alcohols, heparin and heparan.
33. The anti-CCR5 antibody conjugate of claim 32, wherein the polyalkylene ether is polyethylene glycol (PEG) or a derivative thereof.
34. The anti-CCR5 antibody conjugate of claim 33, wherein at least one PEG has an average molecular weight of at least 20 kD.
35. The anti-CCR5 antibody conjugate of claim 31, wherein the apparent size of the conjugate is at least about 500 kD.

36. The anti-CCR5 antibody conjugate of claim 31, wherein the conjugate has at least one of an increase in serum half-life, an increase in mean residence time in the circulation and a decrease in serum clearance rate, compared to a nonconjugated anti-CCR5 antibody or fragment thereof.
37. A method of inhibiting infection of a CCR5+ cell by HIV-1, which method comprises administering to a subject at risk of HIV-1 infection the conjugate of claim 31 in an amount and under conditions effective to inhibit infection of CCR5+ cells of said subject by HIV-1.
38. A method of treating an HIV-1 infection in a subject, which method comprises administering to an HIV-1-infected subject the conjugate of claim 31 in an amount and under conditions effective to treat the subject's HIV-1 infection.
39. The method of claim 38, wherein the amount of the conjugate is effective in reducing a viral load in the subject.
40. The method of claim 38, wherein the amount of the conjugate is effective in increasing a CD4+ cell count in the subject.
41. The method of claim 38, which further comprises administering to said subject at least one conventional anti-viral agent.
42. The method of claim 37 or 38, wherein the conjugate is administered to the subject by a

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method selected from the group consisting of intravenous, intramuscular and subcutaneous means.

43. The method of claim 37 or 38, wherein the conjugate is administered continuously to said subject.
44. The method of claim 37 or 38, wherein the conjugate is administered at predetermined periodic intervals to said subject.
45. The method of claim 37 or 38, which further comprises labeling the conjugate with a detectable marker.
46. The method of claim 45, wherein the detectable marker is a radioactive or a fluorescent marker.
47. A transformed host cell comprising at least two vectors, at least one vector comprising a nucleic acid sequence encoding heavy chains of an anti-CCR5 antibody, and at least one vector comprising a nucleic acid sequence encoding light chains of the anti-CCR5 antibody, wherein the anti-CCR5 antibody comprises two heavy chains having the amino acid sequence set forth in SEQ ID NO:9, and two light chains having the amino acid sequence set forth in SEQ ID NO:6.
48. A transformed host cell comprising at least two vectors, at least one vector comprising a nucleic acid sequence encoding heavy chains of an anti-CCR5 antibody, and at least one vector comprising a nucleic acid sequence encoding light chains of the anti-CCR5 antibody, wherein the anti-CCR5 antibody comprises two heavy chains having the amino acid sequence set forth in SEQ ID NO:12, and

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two light chains having the amino acid sequence set forth in SEQ ID NO:6.

49. The transformed host cell of claim 47 or 48, wherein the cell is a mammalian cell.
50. The transformed host cell of claim 49 wherein the cell is a COS cell, a CHO cell or a myeloma cell.
51. The transformed host cell of claim 47 or 48, wherein the cell secretes the anti-CCR5 antibody.
52. The transformed host cell of claim 47, wherein the vector encoding heavy chains is designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098).
53. The transformed host cell of claim 48, wherein the vector encoding heavy chains is designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099).
54. The transformed host cell of claim 47 or 48, wherein the vector encoding light chains is designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097).
55. The transformed host cell of claim 47, wherein the vector encoding heavy chains is designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) and the vector encoding light chains is designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097).
56. The transformed host cell of claim 48, wherein the vector encoding the heavy chains is designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit

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Designation PTA-4099) and the vector encoding light chains is designated pVK-HuPRO140-VK (ATCC Deposit Designation PTA-4097).

57. The transformed host cell of claim 47, wherein the nucleic acid sequence encoding heavy chains has the nucleic acid sequence set forth in SEQ. ID NO:8.
58. The transformed host cell of claim 48, wherein the nucleic acid sequence encoding heavy chains has the nucleic acid sequence set forth in SEQ ID NO:11.
59. The transformed host cell of claim 47 or 48 wherein the nucleic acid sequence encoding light chains has the nucleic acid sequence set forth in SEQ ID NO:5.
60. A vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:9.
61. The vector of claim 60, wherein the vector is designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation No. PTA-4098).
62. A vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:12.
63. The vector of claim 62, wherein the vector is designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation No. PTA-4099).



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64.A vector comprising a nucleic acid sequence encoding a light chain of an anti-CCR5 antibody, wherein the light chain comprises the amino acid sequence set forth in SEQ ID NO:6.

65.The vector of claim 64, wherein the vector is designated pVK:HuPRO140-VK (ATCC Deposit Designation No. PTA-4097).

66.A process for producing an anti-CCR5 antibody which comprises culturing a host cell containing therein (i) a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:PRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099) under conditions permitting the production of an antibody comprising two light chains encoded by the plasmid designated pVK:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4097) and two heavy chains encoded either by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or by the plasmid designated pVg1:HuPRO 140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), so as to thereby produce an anti-CCR5 antibody.

67.A process for producing an anti-CCR5 antibody which comprises:

- a) transforming a host cell with (i) a plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097), and (ii) either a plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or a plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099); and

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b) culturing the transformed host cell under conditions permitting production of an antibody comprising two light chains encoded by the plasmid designated pVK:HuPRO140-VK (ATCC Deposit Designation PTA-4097) and two heavy chains encoded either by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098) or by the plasmid designated pVg1:HuPRO140 (mut B+D+I)-VH (ATCC Deposit Designation PTA-4099), so as to thereby produce an anti-CCR5 antibody.

68. The method of claim 66 or 67, which further comprises recovering the anti-CCR5 antibody so produced in isolated form.

69. The method of claim 66 or 67, wherein the host cell is a mammalian cell.

70. The method of claim 69, wherein the mammalian host cell is a COS cell, a CHO cell or a myeloma cell.

71. The method of claim 66 or 67, wherein the heavy chains of the anti-CCR5 antibody are encoded by the plasmid designated pVg1:HuPRO140 HG2-VH (ATCC Deposit Designation PTA-4098).

72. The method of claim 66 or 67, wherein the heavy chains of the anti-CCR5 antibody are encoded by the plasmid designated pVg1:HuPRO140 (mut B+D+I) (ATCC Deposit Designation PTA-4099).

73. A kit for use in a process of producing an anti-CCR5 antibody comprising:

a) a vector comprising a nucleic acid sequence encoding a light chain of an anti-CCR5 antibody, wherein the light chain comprises the

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amino acid sequence set forth in SEQ ID NO:6;  
and

- b) a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:9, or a vector comprising a nucleic acid sequence encoding a heavy chain of an anti-CCR5 antibody, wherein the heavy chain comprises the amino acid sequence set forth in SEQ ID NO:12.

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FIGURE 1

Table 1

mAb	Mean Fluorescence Intensity		% Gated	
	L1.2-CCR5	PBMC	L1.2-CCR5	PBMC
mouse IgG1	10	4	1	1
2D7	75	29	92	36
PA8	48	9	73	3
PA9	79	5	96	3
PA10	80	8	96	5
PA11	107	8	96	10
PA12	115	8	96	8
PA14	81	14	96	22

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**FIGURE 2****Table 2**

INHIBITOR COMBINATION	CONCENTRATION RATIO	ASSAY	COMBINATION INDEX	
			90% Inhibition	50% Inhibition
PA12:2D7	10:1	Entry	0.043	0.291
	2:1	Fusion	0.017	0.019
	10:1	Fusion	0.087	0.067
	50:1	Fusion	0.158	0.046
PA12:PA14	10:1	Entry	0.437	0.535
	10:1	Fusion	0.22	0.263
PA14:2D7	1:1	Entry	2.85	1.85
	1:1	Fusion	1.34	1.74
PA12:PA11	1:1	Entry	0.707	0.641
PA12:RANTES	1000:1	Fusion	0.331	0.156
PA14:RANTES	100:1	Fusion	1.6	1.37
2D7:RANTES	100:1	Fusion	0.972	0.962
PA12:CD4-IgG2	10:1	Fusion	0.3	0.31
PA14:CD4-IgG2	1:1	Fusion	0.957	0.566
2D7:CD4-IgG2	1:1	Fusion	1.127	0.302

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FIGURE 3

Table 3

	Epitopes	IC <sub>50</sub> values (μg/ml)			
		cell-cell fusion inhibition	viral entry inhibition	gp120-binding inhibition	calcium flux inhibition
PA8	Nt	-	-	-	-
PA9	Nt/ECL2	-	-	0.24	-
PA10	Nt/ECL2	-	-	0.13	-
PA11	Nt	25.5	-	0.33	-
PA12	Nt	10.0	-	0.24	-
PA14	Nt/ECL2	1.7	.024	1.58	6.4
2D7	ECL2	1.6	.026	1.38	45

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FIGURE 4

	1	5	10	15	20	25	30																							
	M	D	V	Q	V	S	S	P	I	Y	D	I	N	Y	T	S	E	P	C	Q	K	I	N	K	Q	I	A	A	R	
2D7	92	54	90	nd	83	63	82	nd	46	40	90	90	86	111	49	31	45	44	37	52	37	92								
PA8	111	63	78	nd	52	56	32	nd	4	1	53	1	45	100	97	32	36	44	37	34	26	18								
PA9	115	13	0	nd	111	0	13	nd	10	0	19	67	32	39	71	36	32	36	45	30	55	18	111							
PA10	21	14	0	nd	111	59	22	nd	14	3	15	11	48	99	94	27	47	45	45	39	53	13	113							
PA11	70	32	0	nd	111	80	81	nd	40	21	51	74	70	46	85	32	47	36	48	40	47	2	114							
PA12	68	30	0	nd	76	81	51	nd	34	12	49	76	64	40	86	30	41	49	46	34	54	12	93							
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ECL1

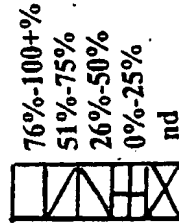
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PA8	38	92				49	118	32	113		96	86	64	0	39
PA9	31	66				27	81	16	119		108	19	31	0	41
PA10	67	83				36	109	0	113		112	97	81	0	40
PA11	39	81				24	91	25	78		60	94	71	0	60
PA12	61	67				35	91	29	77		76	79	102	35	33
PA14	75	79				63	83	23	86		47	89	80	7	77

ECL2

	170					175					180					185					190						
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2D7	62	55	17	0				62	64	65	0	83	95	51	31	37	60	92	71	41	34	71	39	37	45	103	
PA8																											
PA9	38	40	38	11				65	51	81	26	90	100	51	41	46	90	94	38	38	93	31	40	37	62		
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PA12	31	34	38	31				32	103	114	8	71	85	49	27	101	90	85	34	34	34	34	34	34	34	34	
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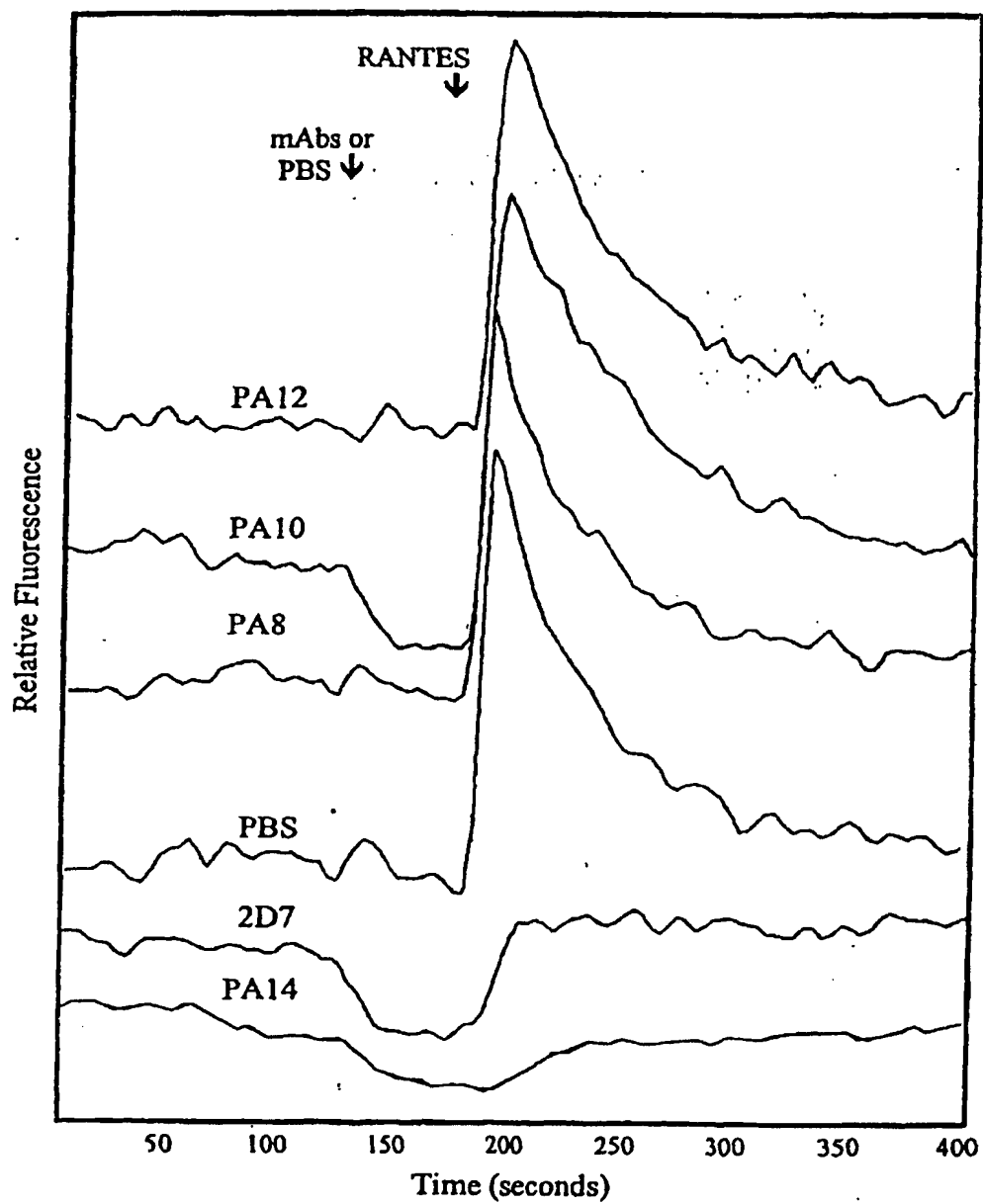
ECL3

	265										270			275			
	Q	E	F	V	G	L	N	N	C	S	S	S	N	R	L	D	Q
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PA8	×	78	25	0			37	43	47	85	71	73	86	99	×		74
PA9	×	32	25	0			67	40	50	71	81	72	90	93	×		113
PA10	×	47	25	0			64	60	56	93	103	49	74	72	×		118
PA11	×	60	28	0			35	60	49	78	71	70	72	74	×		164
PA12	×	60	28	0			34	36	46	83	72	99	82	78	×		111
PA14	×	92	82	42			80	31	92	111	90	98	74	78	×		113



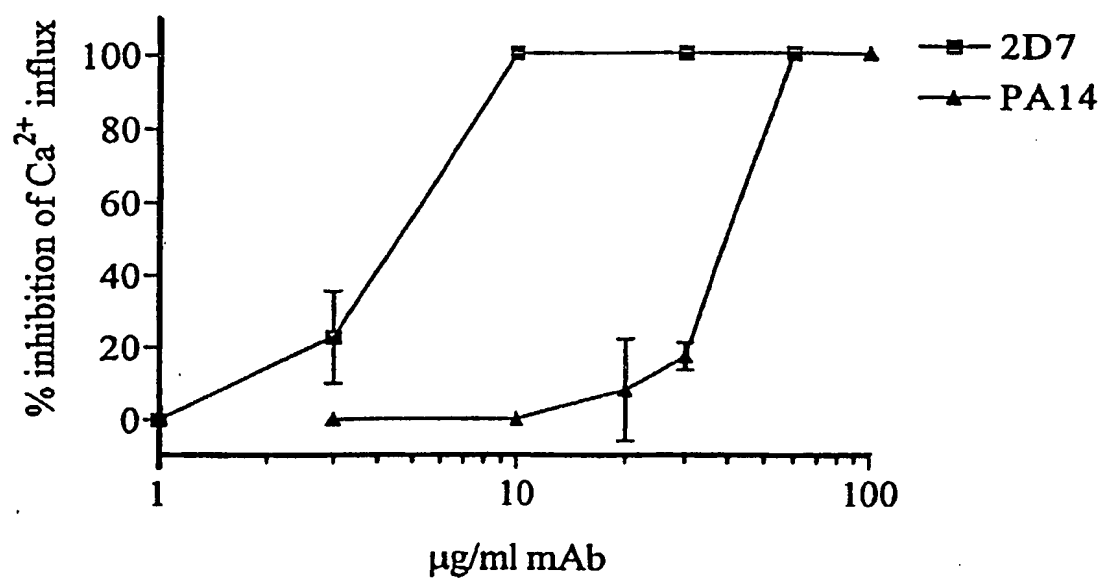
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FIGURE 5A

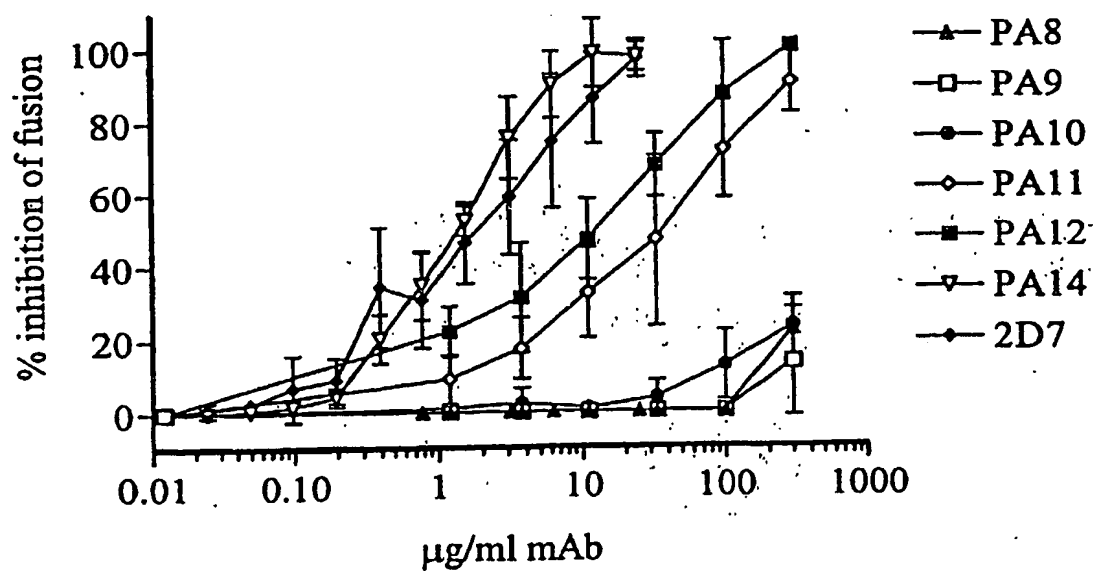




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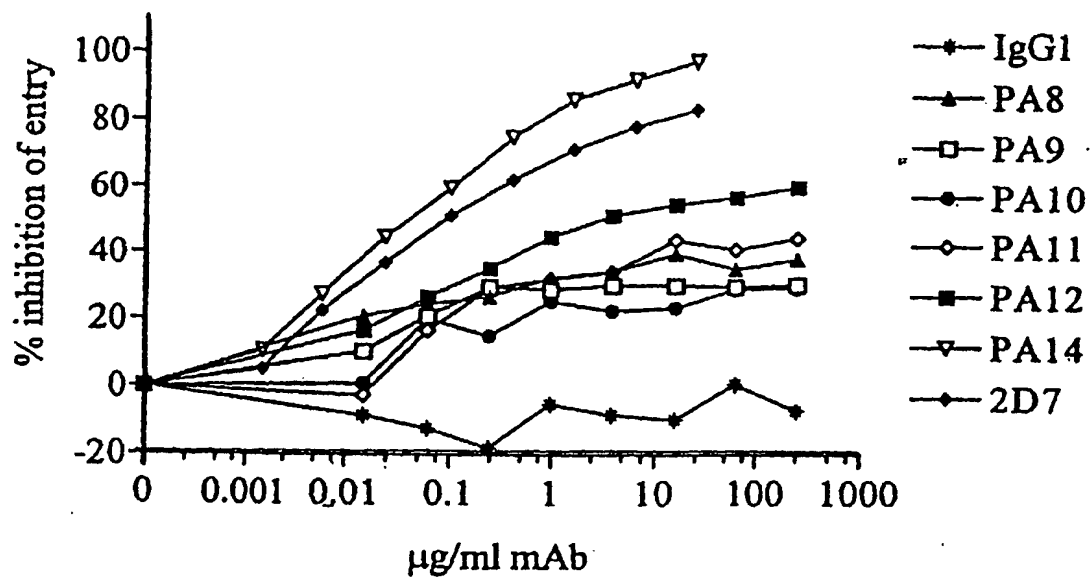
**FIGURE 5B**

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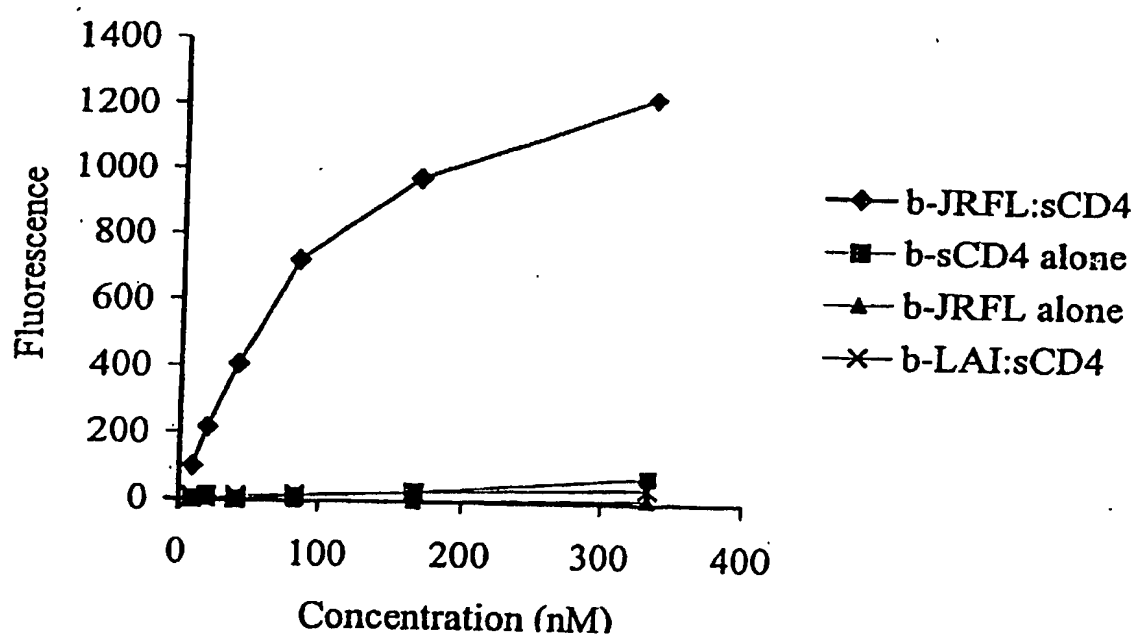
**FIGURE 6A**

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FIGURE 6B

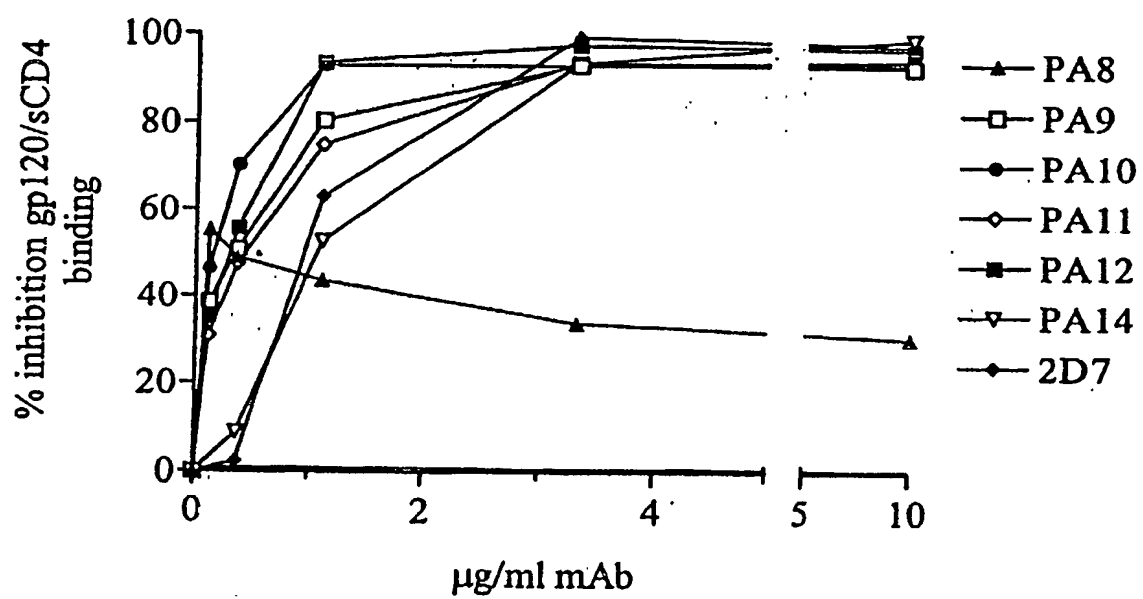


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**FIGURE 6C**

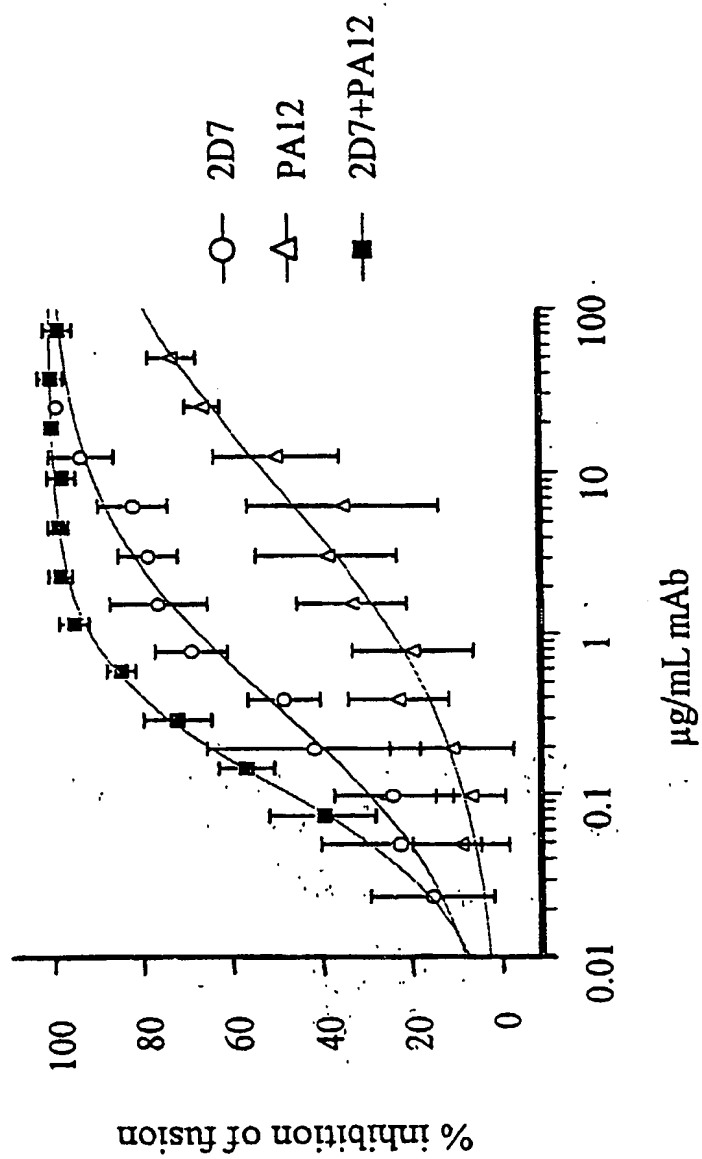
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FIGURE 6D



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FIGURE 7



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FIGURE 8

30  
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 M K L P V R L L V L M F W I P A  
 60  
 90  
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 S S D I V M T Q S P L S L P V T P G E  
 120  
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 P A S I S C R S S O R L L L S S Y G H T Y  
 180  
 210  
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 L H W Y L Q K P G Q S P Q L L I Y E V R  
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 270  
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 390  
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 429  
 AACTCTAGA

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## FIGURE 9

30 59  
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M E W S G V F I F L L S V T A G

89 118  
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V H S E V Q L V E S G G G L V K P G G S

148 177  
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L R L S C A A S G Y T F S N Y W I G W

207 236  
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V R Q A P G K G L E W I G D I Y P G G N

266 295  
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Y I R N N E K F K D K T T L S A D T S K

325 354  
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384 413  
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C G S S F G S N Y V F A W F T Y W G Q G

443 457  
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T L V T V S S



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**FIGURE 10**

30 60  
TCTAGACCACCATGGAATGGAGCGGGGTCTTTATCTTTCTCCTGTCAGTAACTGCAGGTG  
M E W S G V F I F L L S V T A G

90 120  
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V H S Q V Q L V Q S G P D V K K P G T S

150 180  
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M K M S C K T S G Y T F S N Y W I G W V

210 240  
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R Q A P G Q G L E W I G D I Y P G G N Y

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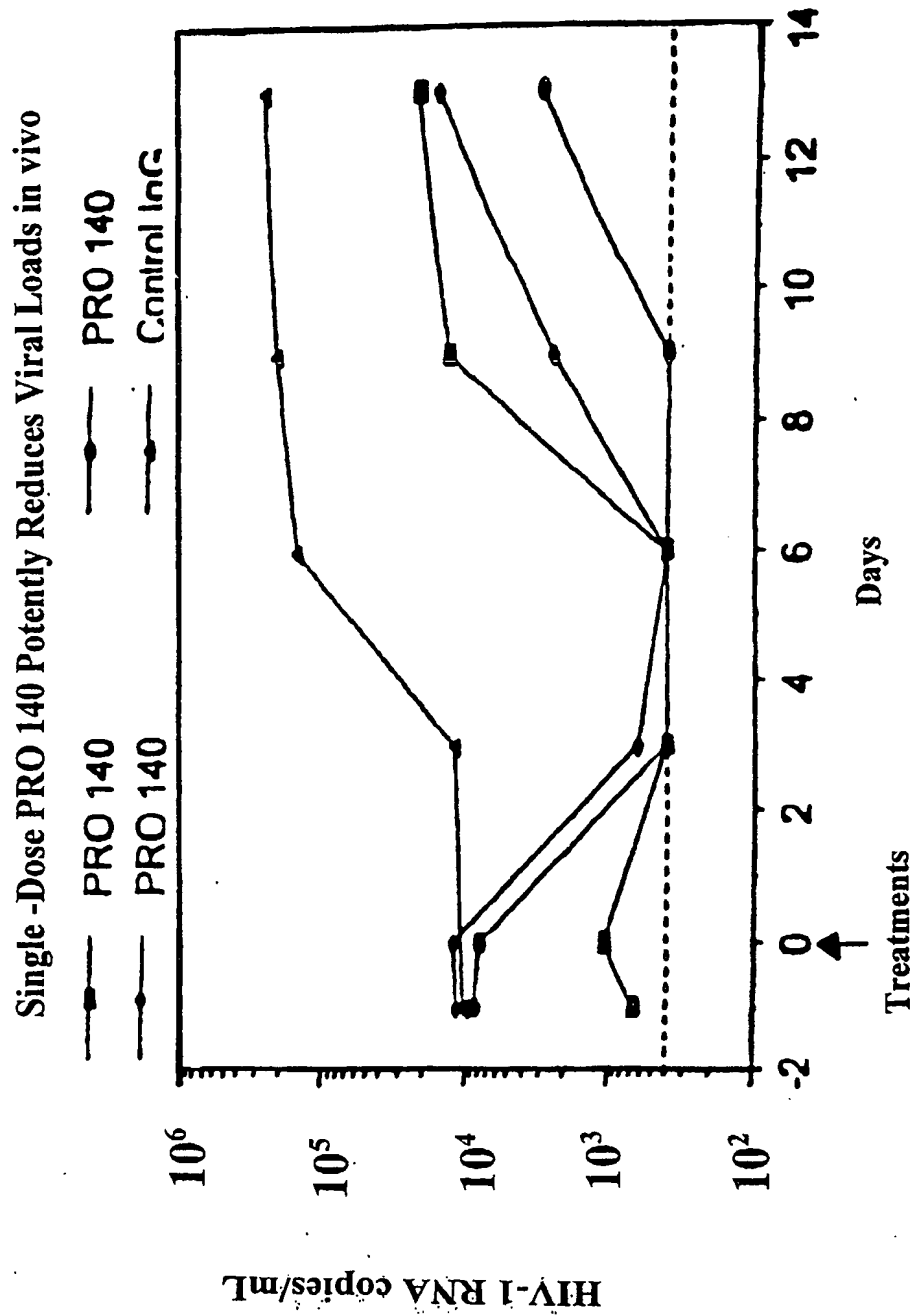
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V T V S S

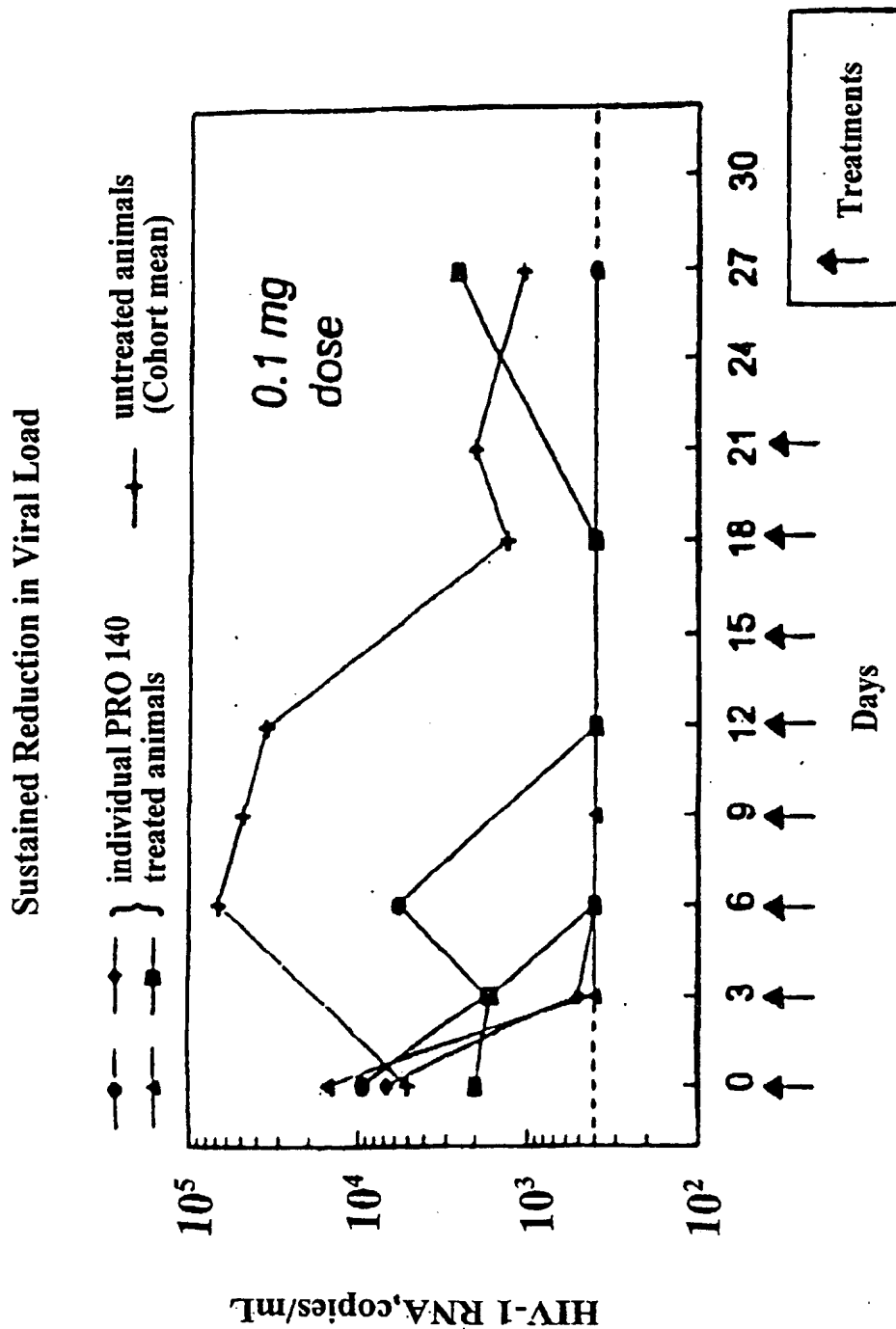
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FIGURE 11



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FIGURE 12



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FIGURE 13

No Depletion of Lymphocytes			
Percent of circulating lymphocytes*			
	CD4 + cells	CD8 + cells	human cells
PRO 140	8.9	8.3	34
treated animals			
untreated	0.7	4.8	19
animals			

\*Cobort mean values measured at Day 30 (9 days post-treatment)

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FIGURE 14

Humanized PRO 140 Potently Blocks CCR5-mediated HIV-1 Cell-Cell

Fusion

PRO 140 antibody	number of assays	Median Inhibitory Conc., $\mu\text{g/mL}$ IC <sub>50</sub>	IC <sub>90</sub>
murine	6	0.99	5.90
humanized PRO 140 #1	6	2.55	14.01
humanized PRO 140#2	6	2.24	8.55

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FIGURE 15

Humanized PRO140 Mediates Potent, Subtype-Independent Inhibition of HIV-1

	number of replicate assays per virus	median IC <sub>50</sub> µg/mL virus (genetic subtype)		
		JR-FL (B)	Case C 1/85 (B)	DJ258 (A) DU151 (C) overall
murine PRO 140	≥9	2.1	3.7	9.7 1.6 2.9
humanized PRO140#1	≥9	2.9	7.4	15 14 11
humanized PRO 140#2	8	2.4	3.9	3.2 4.7 3.6

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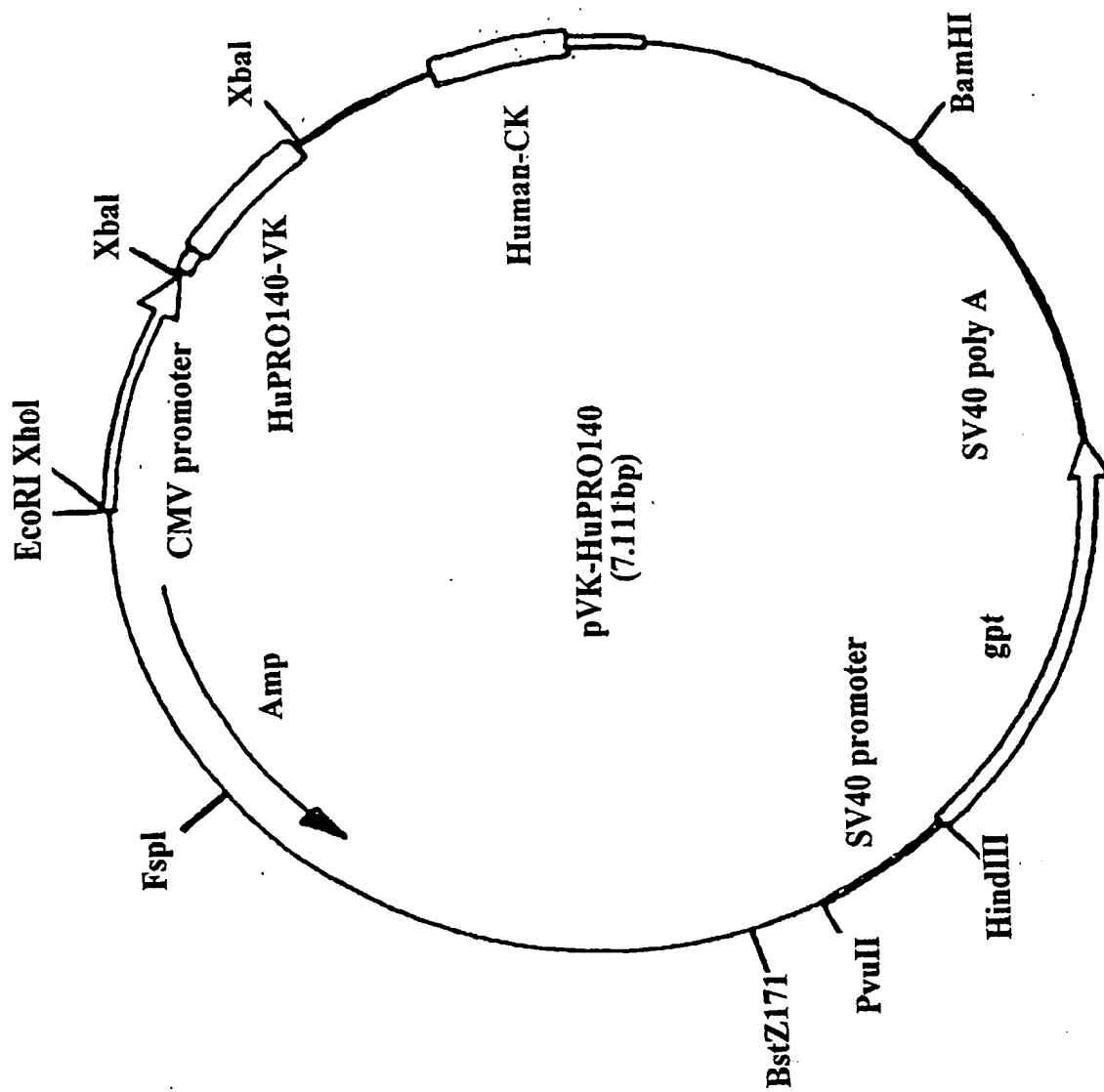
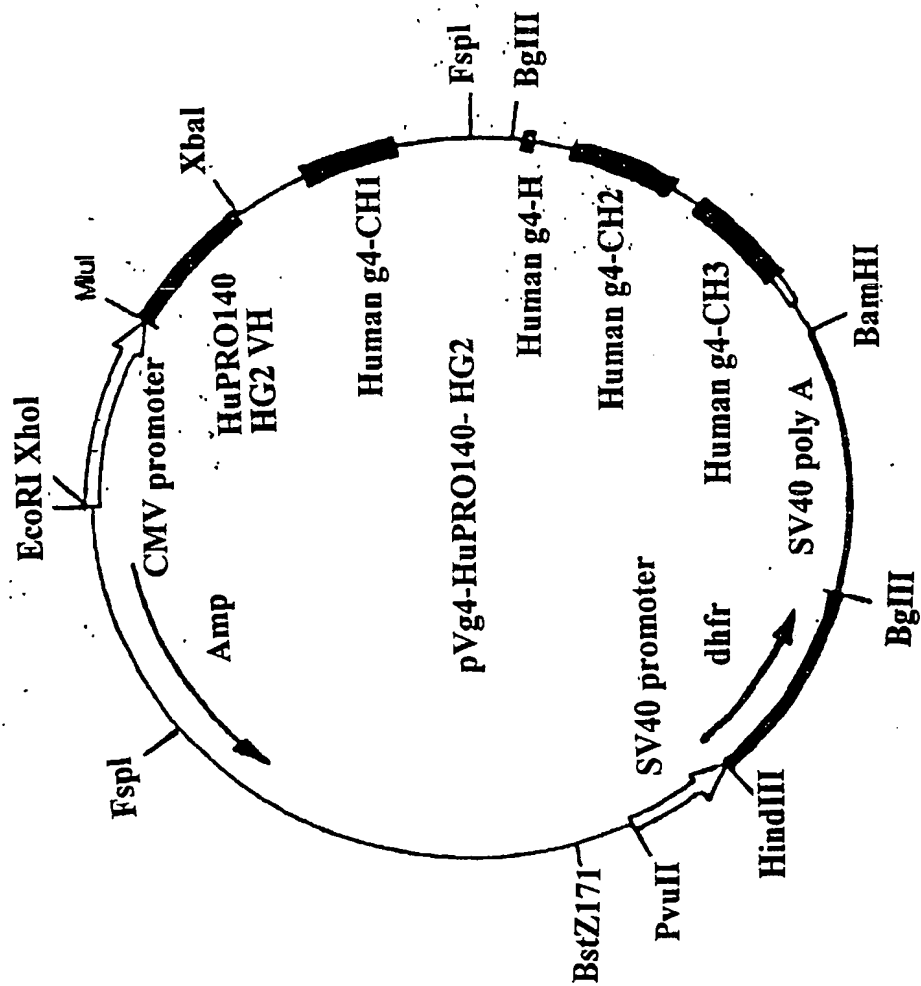


FIGURE 16

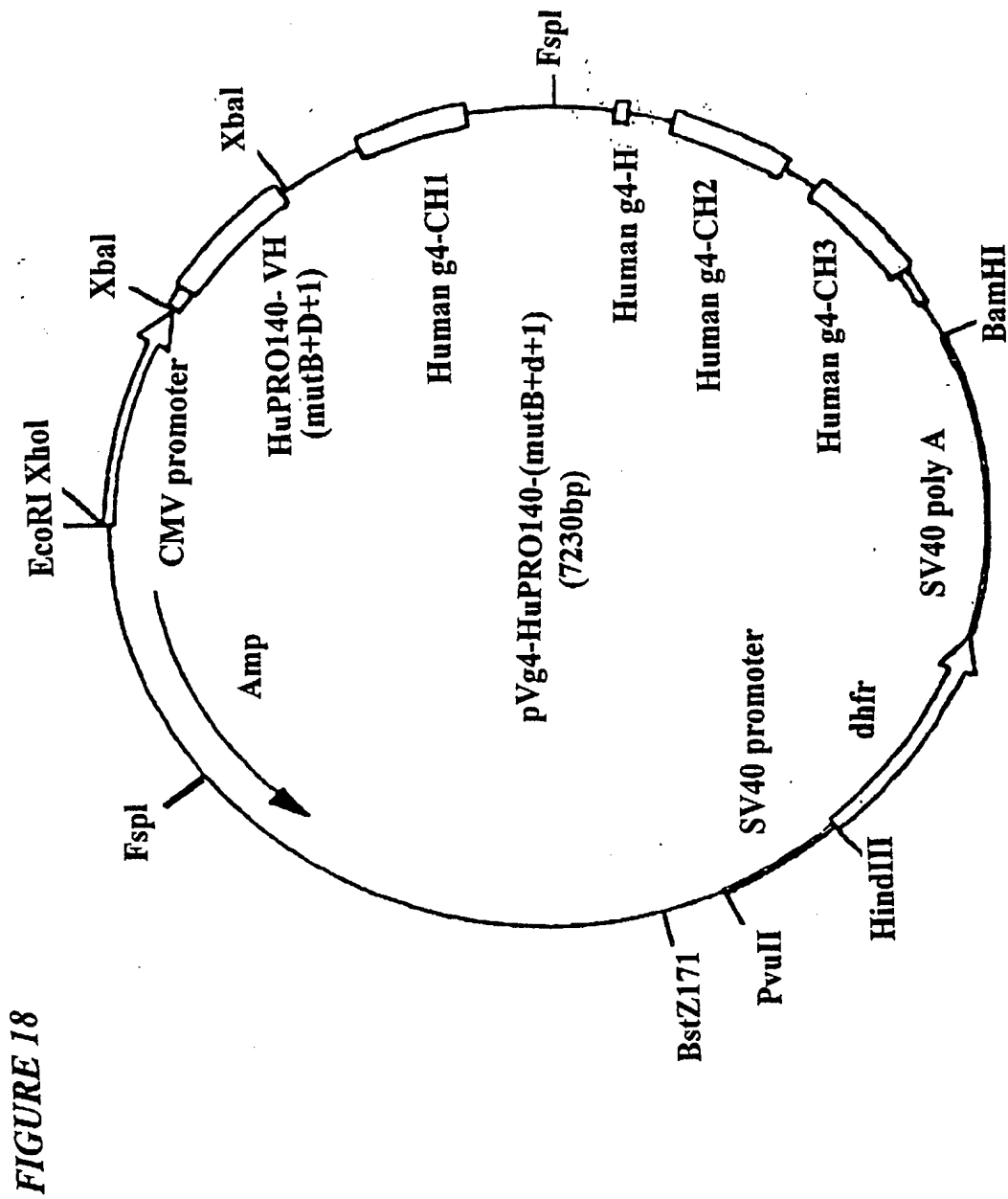
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FIGURE 17





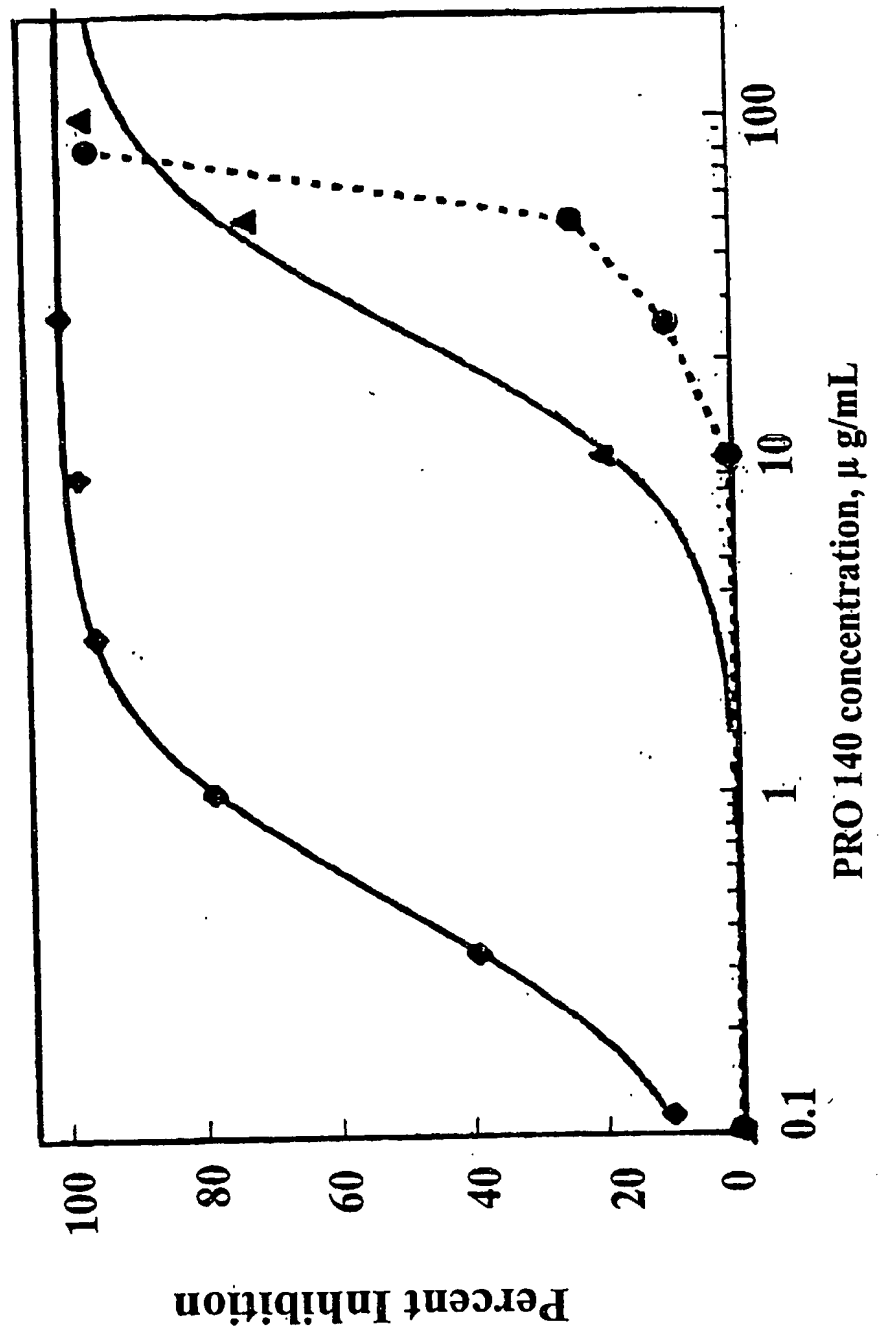
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**FIGURE 19** hu PRO 140 Blocks HIV-1 BUT Not RANTES Signaling

◆ huPRO 140 HIV-1    ▲ muPRO 140 RANTES    ● huPRO 140 RANTES



## SEQUENCE LISTING

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Glu Trp Ile Gly Asp Ile Tyr Pro Gly Gly Asn Tyr Ile Arg Asn Asn  
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Glu Trp Ile Gly Asp Ile Tyr Pro Gly Gly Asn Tyr Ile Arg Asn Asn  
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Glu Lys Phe Lys Asp Lys Thr Thr Leu Thr Ala Asp Thr Ser Thr Ser  
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 tca 423



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/05500

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : C12N 5/06 US CL : 435/328 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/85.1, 130.1, 134.1; 435/320.1, 326, 328; 530/388.1, 388.22; 536/23.4, 23.53  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, E --- A, E	US 6,528,625 B1 (WU et al) 04 March 2003 (04.03.2003) see claims.	1-5, 20-46, 66-73 ----- 6-19, 47-65
Y --- A	CO et al. Humanized Antibodies for Antiviral Therapy. Proceedings of the National Academy of Science, USA. April 1991, Vol. 88, pages 2859-2873, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65
Y --- A	TRKOLA et al. Potent, broad-spectrum inhibition of human immunodeficiency virus type 1 by the CCR5 monoclonal antibody PRO 140. Journal of Virology. January 2001, Vol. 75, No. 2, pages 579-588, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65
Y --- A	OLSON et al. Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. Journal of Virology. May 1999, Vol. 73, No. 5, pages 4145-4155, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
<b>* Special categories of cited documents:</b>		
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"B" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 10 July 2003 (10.07.2003)	Date of mailing of the international search report <b>18 AUG 2003</b>	
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer <i>Ulrike Winkler</i> Telephone No. 703-308-0196	

## INTERNATIONAL SEARCH REPORT

PCT/US03/05500

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y --- A	PARREN et al. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. <i>Journal of Virology</i> . September 2001, Vol. 75, No. 17, pages 8340-8347, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65
Y --- A	LEHNER et al. Immunogenicity of the extracellular domains of C-C chemokine receptor 5 and the in vitro effects on simian immunodeficiency virus or HIV infectivity. <i>Journal of Immunology</i> . January 2001, Vol. 166, No. 12, pages 7446-7455, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65
Y --- A	WU et al. CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. <i>Journal of Experimental Medicine</i> . 05 May 1997, Vol. 185, No. 9, pages 1681-1691, entire document.	1-5, 20-46, 66-73 ----- 6-19, 47-65

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/05500

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
  2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
- Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

PCT/US03/05500

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, 20-46 and 66-73, drawn to an antibody and a method of making and using the antibody.

Group II, claim(s) 6-19 and 47-65, drawn to a nucleic acid and transformed host cell.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because under unity of invention between different categories of inventions will only be found to exist if specific combinations of inventions are present. Those combinations include:

- A) A product and a special process of manufacture of said product.
- B) A product and a process of use of said product.
- C) A product, a special process of manufacture of said product, and a process of use of said product.
- D) A process and an apparatus specially designed to carry out said process.
- E) A product, a special process of manufacture of said product, and an apparatus specially designed to carry out said process.

The allowed combinations do not include multiple products, multiple methods of using said products, and a method of making a product as claimed in the instant application.

In the instant case the antibodies of Group I are made up of amino acids which have different structure and different function from the nucleic acids of Group II.

Accordingly, Groups I and II are not so linked as to form a single general inventive concept and restriction is proper.

### Continuation of B. FIELDS SEARCHED Item 3:

WEST, DERWENT, STN-BIOSIS, MEDLINE, EMBASE

antibody, monoclonal, CC chemokine receptor 5, CC-CKR5, CKR5 receptors, CCR5 receptor, inventor search.